Answer to reviewers.

All comments have been addressed according to reviewer's suggestions. We have also included comments in answer in blue to all the questions raised by each reviewer. All changes to our manuscript are specified in "manuscript with track changes" to assist the Editors and Reviewers in evaluating our revised manuscript.

We do hope that this manuscript will now be suitable for recommendation in PCI Animal Science

We look forward to hearing from you.

Yours sincerely,

Dr. C.Hue-Beauvais

Reviewer 1 : Marion Boutinaud, 23 Mar 2022 09:58

Abstract

L29-30 the sentence is not clear to me, what is the problem with the common practices during rearing in commercial rabbit production

Rabbits does are raised in the same conditions as the animals intended for the meat production. However, these feeding strategies, including restriction are not optimal for further reproductive and lactation capacities.

L33 the authors analyzed 4 strategies with two periods of restriction. Please modify the text

"high or moderate restriction feeding applied from 5 to 9 weeks of age and/or restricted or *ad libitum* over the following 3 weeks constituting the pubertal period"

Please check the English language for example L39-40-43 please use the past tense: have/ had vary / varied, have impacted /impacted; and L336 "compared with" instead of" compared to "…

Changed in the manuscript

L39 what kind of reproductive parameters were measured in this paper?

We numbered the number and the viability of fetuses by females à mid-pregnancy.

L40-L41 and L43 be more specific about the results obtained. What were the effects?

"vary" was replaced by "decreased" and "modified" replaced by "increased" in the text.

L42 When did you collect the mammary tissue samples? At what period the gene expression was studied? This should be indicated in the abstract.

"at mid-pregnancy" added in the manuscript

L43 did you perform the analyses in the mammary epithelial cells or in the mammary tissue ? Did you isolate mammary epithelial cells?

"Cells" was replaced by "tissue"

Please indicate the p-value when the effect is significant L41-46

($p \leq 0.05$) was added in the abstract

L45 what does mean regarding further lactation?

Further lactation refers to the continuation of putative or actual lactation in relation to the stage of pregnancy studied.

L479 front my point of view, the conclusion is not clear enough about what is the best strategy for the mammary gland development.

We show that nutritional strategies with the least restriction are the most suitable for breast development. Nevertheless, currently, breeders raise rabbit does in the same premises and with the same feeding (qualitatively and quantitatively) as meat-producing animals, mainly for financial reasons. This work was initiated by the companies CCPA and Hypharm to be a support of advice for the actors of the sector.

L60-61 The expression "reared the same way as fattening animals" is not clear to me

That means that breeders raised rabbit does in the same facilities and with the same feeding (same diet and same feeding strategy) as meat-producing animals.

L64 kit survival. Please remove "s" to "kits"

Corrected in the manuscript.

L91-L93 as previously written in the introduction part, the effect of restricted feeding strategy is known in gilts and goats. Please indicate here the rabbit specie

Corrected in the manuscript.

L111 the number for the authorization from the French Ministry of Agriculture should be mentioned here.

Rabbit were raised at Hypharm which is a breeder/selector skilled in using different nutritional strategies. Females were transported to INRAE only for euthanasia and post-mortem sampling, which does not require an authorization from the French Ministry of Agriculture. Nevertheless, the local ethics committee had been consulted during the drafting of the project between partners but without attribution of a registration number.

L118-120 Please clarify the amount of feed given to the does in the SR Group. According to Fig 1 it depends on the live weight. This information is lacking in the text.

Requested information has been added to the text.

L120 I may did not well understand but I suggest to change the text to "from 9 to 11" instead of "12".

Corrected in the manuscript

L128 the method of sacrifice should be described here.

"euthanized by exsanguination" was added in the text

L129 why a fasting has been performed before the sacrifice? This has been explained later in the text, but should be clarified in that section.

"allowing subsequent blood metabolic assays." was added in the text in that section.

L129 what is a hydrous fasting? The remove of food and water? of only water or only food?

An hydrous fasting consists of removing food while allowing *ad libitum* access to water in order to avoid any dehydration that could be responsible for the suffering of the rabbit and also for a disturbance of the blood metabolic parameters.

L133 the method of sacrifice should be mentioned in the previous paragraph.

"euthanized by exsanguination" was added in the text in the M&M section.

L136-139 did you measure the weight of the excised mammary tissue? Maybe this information could give an idea of the mammary development?

In rabbits, as in other mammals (including women), the weight or size of the mammary gland at mid-pregnancy does not correlate with the development of epithelial tissue, nor with subsequent lactation capacity.

L147 I did not well understand the number of sample per rabbit. In the previous paragraph, it is mentioned that only left inguinal glands were excised.

2 samples per left mammary gland was fixed for histology, and 4 sections separated at least by 100 µm per sample was processed. This sampling was designed to allows a representative histological analysis of the mammary gland development at mid-pregnancy.

L148 the percentage of areas

Modified in the text

L166 only one gene was used as a reference gene. Nowadays at least 2 reference genes are asked. Did you check if the expression of Tbp gene was stable according with your different groups of animals?

In previous work we tested 3 household genes in mammary tissue (TBP, CPR2 and GAPDH). At G14 in rabbits only TBP and CPR2 are stable and have comparable expression levels in the mammary gland, through the different groups. We therefore chose TBP, already used in previously published work (Hue-Beauvais et al. Dev Dyn. 2019 doi: 10.1002/dvdy.91).

L175-179 The statistical analyze should be completely revised. Since you performed two strategies of feed restriction one during post weaning and one during Puberty, you can consider these two strategies as two treatments and you could analyze the effects of both treatments and the interaction of both treatments using an ANOVA. You also need to check if your data can fit or not with this type of analyze (the linearity of the residus and the homogeneity of the variance of the residus).

Here you wrote that you performed either student's t-test of Mann Whitney U test. The authors should indicate what tests have been considered for each variable. When you considered your data according to the effect of time (for live weight), you also should consider the "rabbit effect" as either a repeated effect or a random effect, and the interaction between the time and the two treatments

Statistical analyse has been completely revised, considering the growth analysis between 5 and 18 weeks of age, covering post-weaning and puberty period, using ANOVA tools.

In order to take into account, the longitudinal nature of the data, a linear mixed model was used for using the nlme R package, with a random animal effect and a first order autoregressive correlation structure. For the fixed effects, a linear curve was first considered, with the time effect as continuous. The fixed effects were therefore in this case the group effect, the continuous time and an interaction between group and time. In order to have a more precise analysis of the differences among groups between times we performed a second type of analysis with the time effect as discrete (per week), and the lsmeans option to specify the contrasts of interest.

L193-220 each time a numerical results (L193, 199…) is shown please provide the sem.

There is no sem. for numerical values corresponding to daily food intake for each group : 95g/d for SR (7% of live weight at weaning + 2 g/d) and 120,75 g/d for MR (9% of live weight at weaning + 2,5 g/d). These values were calculated by the breeder from the mean live weight of each group.

L193-212 When the effect is significant or not please provide the P-values (L200, 202, 203, 204, 206, 208, 211, 213, 219, 220…L237, L239, L240, L242, L250, L251)

P-values were added in the text.

L193-L267. When the statistical analyzes will be revised the data should be presented according to the new statistical analyze showing the p-value for the effect of the feed restriction at post weaning, the effect of feed restriction at puberty and the effect of the interaction of both

The results has been modified accordingly to the ANOVA statistical analysis.

Figure 1, 3 , 4, 6 and 7please add a space before and after = (n = 10)

Space was added in figures of Manuscript V2 (not in manuscript with track changes because legend figures are in pdf).

Figure 2-6 please indicate each treatments in the legends as for Figure 1

Explanations of the acronym of each treatment are added in the legends of figures 2 to 7 in manuscript V2 ((not in manuscript with track changes because legend figures are in pdf).

Figure 2 how did you measure the ingestion? This should be indicated in the material and method part

The ingestion was measured in Hypharm facilities by difference between quantity of food distributed and the remaining quantity not ingested. "…By the difference between the weight of the distributed ration and the refusal" was added in the M&M section.

Figure 2 These data should be analyzed including a time effect

Data has been analyzed again including time effect as described in statistical section. Figure 2 has been completely revised with regard to the ANOVA analysis.

Figure 1 The average daily food intake given in the table A should be given with the mean ±SEM. You could test the effect of each treatment on these data and give the p-value of the effect of the feed restriction at post weaning, the one for feed restriction at puberty and the one for the interaction of both. These P values should also be given in the legends of Figure 3, 4, 5 and 6

The figure 1 show the design and the description of the four feeding strategies applied. Food intake values were not shown. Significant P values (<0.05) are given in the legends of figure 3, 4, 5 and 6.

Figure 1 legend. Please add the species "Each group of rabbits"

Added in the legend of figures 1 in manuscript V2 (not in manuscript with track changes because legend figures are in pdf).

Figure 1 table B “Puberty” instead of “Pubertal”

Modified in figures 1 B in manuscript V2 (not in manuscript with track changes because legend figures are in pdf).

Figure 4 a table is probably more appropriate since no effect seems to be observed. A table with the means mean ±SEM and the p-value for the different effects-

Figure 4 was completely revised and presented as a data table combined with statistical analyses of the effect of feeding strategies by time periods.

Figure 5 Please modify the title of the (B). My suggestion “Relative quantification of areas occupied by the different types of mammary tissue”

The (B) title was modified in figures 5 in manuscript V2 (not in manuscript with track changes because legend figures are in pdf).

Figure 5 please add % of area in the Y-axis legend

Y-axis legend was modified in figure 5 in manuscript V2 (not in manuscript with track changes because legend figures are in pdf).

L295 please add as expected before consequently

Added in the manuscript

L311-318 : the blood metabolic parameters have been measured at euthanasia after 12 hours of fasting. In the discussion, the authors should maybe suggest that theses parameters were not measured at the appropriate time to observe the effect of feed restriction. The blood sample collection should have been done during the period of feed restriction and not at D14 of pregnancy when the both feed restriction periods are over. So I don’t agree with the last sentence of the paragraph L317. I wonder if there is any interest in showing these results.

It has been previously shown that nutrition changes may have long term effects on metabolism. In this perspective, we measured metabolic parameters nine weeks after the end of the feeding strategies. The text has been modified to be clearer.

L325 () ?

Removed in the manuscript

L334 since the area occupied by fat and epithelial tissue were considered together you can’t really write that it is due to a combined decrease in fat and epithelial tissues. You don’t know if it is due to fat epithelial or both. Please change this.

We have chosen this combined representation because adipose and epithelial tissue decreased together in our study. Combination allows us to have a powerful statistical result

than each effect taken separately: for adipose tissue SRR (18,95±3,11 %) *vs.* SRAL (12,69±2,07%) P>0,05 and for epithelial tissue SRR (22,71±1,5 %) *vs.* SRAL (20,34±1,92%) P>0,05.

L335-337 in the result part and in figure 5 it is not mentioned that the luminal part is higher in the most restricted animals than in any other groups of animals. Please revise this sentence

The sentence was modified accordingly in the manuscript.

L338-340 The authors seemed to observe an interaction between your feed restriction treatments on the % of area occupied for connective tissue. Could you give some hypothesis using what you know about the period of mammary development (epithelial versus connective tissue during post weaning or pubertal period).

This situation can remind what happen during post-weaning and puberty where the ratio connective tissue /epithelial tissue is in favor of connective one. This phenomenon may also be due to hormonal variations, such as sex steroids which can promote cells proliferation.

L354-355 is the reference Takeuchi et al.2001 really useful here? In Takeuchi et al. they studied the effect of different types of lipid supplementation on the liver whereas the authors here studied the effect of feed restriction on the mammary tissue. So there is no link between both studies

Takeushi *et al.* showed that hepatic enzymes related to lipogenesis are increased in the liver especially in response to diet producing high circulating nutrient levels. We find a comparable phenomenon with a poor restricted diet also allows a high level of circulating nutrients in the whole body and therefore also in tissues. In response, mammary tissue increased the expression of lipogenesis enzymes, such as FasN.

L368 what tend to be increased mean here? With the P-value this will help to understand. This information should be given in the result part and not in the discussion.

P=0,041 was added in the result section concerning the comparison between SRAL and MRAL group for LALBA expression.

L385-L396 this part seems to be disconnected of the results obtained in the study. So do you really need to add this paragraph? The link with the objectives of the study in not clear.

The objective of this study is to estimate if the feeding strategies used to produce broiler rabbits are compatible with the physiological development, and particularly the mammary development of future breeding rabbits.

L397 to conclude what is the most critical period for feed restriction? Using a ANOVA model you could see which period is more critical for mammary development.

The most adverse period depends on which parameters are observed.

L402 your conclusion is that a severe restriction could adversely affect mammary tissue and not health. Please remove health here. Please explain which phase is the most critical and what are the conclusion of your study.

The differences between applied strategies are quantitatively weak and qualitatively inexistent and therefore the observed effects are equally small. Nevertheless, our previous work has shown that few periods of development are critical windows of susceptibility to nutrition, and we see in this study that depending on the parameters measured (growth, metabolism or mammary development), the periods involved are not necessarily the same.

Reviewed by Davi Savietto, 18 Mar 2022 16:52

Line 28: consider changing the word “performance” by “potential”. The reproductive capacity of an animal is, in part, given by the genetic potential of each individual. By modulating the environmental conditions, such as the feed, the expression of the genetic potential is altered.

Modified in the text

Lines 29-31: please revise this sentence. Selected and non-selected rabbits have nutritional needs. What may have changed through selection for high prolificacy (and other reproductive traits) is the nutritional requirement. In respect to the feeding strategy during the rearing period, this sentence introduces the hypothesis that selection for reproductive traits also changed (incremented) the nutritional requirements of young rabbit females. This hypothesis seem to not be sustained by the available literature (e.g. compare the H1M & H2M groups in the paper of Quevedo et al., 2005; https://doi.org/10.1079/ASC40850161).

The word "specific" has been added to the sentence for introducing the idea that the nutritional strategies used to produce broiler rabbits may not be optimal for the development of reproductive potential but especially for mammary development, according to some of our previous work illustrating the influence of puberty as a window of susceptibility to feeding and also the work of Read (doi: 10.1002/dvdy.91, doi: 10.2527/jas.2016-0678).

Lines 32-35: please introduce the objective of the study. The feeding strategies are the means to attain the objective. Please consider the following suggestion: “The aim of this study was to analyse the impact of four different feeding strategies in the early life of rabbit females (combination of high or moderate feed restriction from 5 to 9 weeks of age with restricted or ad libitum feeding regime from 9 to 12 weeks of age), on their…”

The manuscript was modified accordingly.

Lines 37-38: please mention the period of the measurements. Is it only from 3 to 9, from 3 to 12, from 9 to 12 weeks or other periods? Lines 38-40: same as above. Line 46: introduce the word “feed” at “…restrictive feed strategies…”

The period of measurement was added by “mid-pregnancy” and the period of “puberty” was specified.

Lines 47-49: Did diets affect milk yield, quality and neonatal survival? If not, please review this recommendation.

Our analyses were performed at 14 days of gestation, which did not allow us to measure the effects of mammary development on lactation. However, previous studies have shown that a change in mammary development at mid-gestation had an impact on milk quality and therefore on pup development.

INTRODUCTION

Lines 54-55: A reference is required.

The following reference wad added in the manuscript : Cartuche et a;. 2014

Lines 55-56: Dietary restriction is mainly used to reduce the risk of digestive disorders in the post-weaning period (cited review). The reduction of feed costs is a collateral, non-negligible, consequence of this strategy related to the capacity of young rabbits to re-gain the live weight not acquired during the restriction period. Based in this information and to correctly quote the references, please re-structure this paragraph (lines 54-59).

The paragraph was modified accordingly.

Line 62: consider changing the words “control” by “restricted”.

Suggested modification was made in the text.

Lines 63-64: please specify the periods and mention the values for the reduced fertility compared with the ad libitum strategy. “Feed restriction during…energy deficit during…, leading to a reduced fertility (XX vs YY; Pascual et al. 2003)”. The reference provided (if Pascual JJ, Cervera C, Blas E, Fernández-Carmona J. 2003. High-energy diets for reproductive rabbit does: effect of energy source. Nutrition Abstracts and Reviews, 73 (5), 27R-39R) is not the most adequate to sustain this argument. This reference is not listed in the Reference section. Please revise this issue.

Indeed, the reference Pascual 2003 did not correspond to the works showing the effects of food restriction on the reproductive performance of rabbits. This reference has been replaced in the following paragraph which refers to studies on deregulated feeding. The cited works (Cappon 2055 and Matsuoka 2006) showed that feeding restrictions, particularly during gestation, has induced deleterious effects on the survival and development of the offspring.

Lines 64-65: revise this sentence. Martinez-Paredes et al. (2019) observed no effect of a feed restriction plan (CR diet) when compared to the ad libitum strategy (CAL diet) on milk yield and composition (1st lactation), blood metabolites (females), kits live weight (1st lactation), kits mortality (1st lactation), kits feed intake (1st lactation), females reproductive interval (1st to 2nd parturition), or in any other measured parameters.

Errors in the Endnote database of references resulted in inadequate citations for some paragraphs. Erroneous references or references that do not correspond to the paragraphs have been removed and replaced with the appropriate references. Our previous work on effect of deregulated diet on milk composition and long-term effect on pups has been added.

Lines 70-71: consider the following change: "…are long-term process that starts early in life and continues through adulthood…"

Suggested modification has been added in the manuscript.

Lines 71-73: mention the early life factors that influence the mammary gland development in the pre-pubertal phase. Of the mentioned factors to what extent, does dietary restriction impede the mammary gland development? Provide key references to sustain this argument. The cited reference (Robinson 1995) may not be the most suitable here. Lines 75-76: please provide a reference. See the comment above.

The sentence has been modified to introduce the notion that early factors of any kind may have the capacity to alter long-term cell development and functionality. The impact of nutrition (restriction, modification, ...) on mammary development is then included in the text and a journal reference has been added.

Line 93: delete the word "still" Recommendation: authors may restructure and shorten the introduction section. They may focus on the physiological changes in the mammary gland development induced by an unbalanced intake of nutrients (too much and/or too little) in the early life of mammals. Then introduce the potential issue of feed restriction strategy adopted in rabbit production, a strategy that focus on the correct body development and on the reduction of fat deposits (adequate body composition) at first insemination, on the subsequent development of the mammary gland. It is important to mention that although, the available literature does not support the hypothesis that feed restriction impairs the milk yield or quality (Martinez-Paredes et al., 2019), there is a lack on the knowledge on the impacts of a long-term feed restriction, starting as soon as 5 weeks old, on the physiological development of the mammary gland and tissues. Then present the objectives of the study.

The last paragraph of the introduction has been modified as suggested to introduce our work in a more nuanced research context. The reference previously cited has been inserted in this paragraph.

MATERIAL AND METHODS

Lines 109-111: Provide the agreement number issue by the French Ministry of Education, Research and Innovation (MESRI).

Rabbit were raised at Hypharm which is a breeder/selector skilled in using different nutritional strategies. Females were transported to INRAE only for euthanasia and post-mortem sampling, which does not require an authorization from the French Ministry of Agriculture. Nevertheless, the local ethics committee had been consulted during the drafting of the project between partners but without attribution of a registration number.

Lines 113-114: Provide the temperature range and average measured during the entire experimental period. Even in indoor conditions, a constant temperature of 18°C is the target temperature. Concerning the illumination program, provide the numbers of dark and light hours at each period.

The rabbits were raised on Hypharm's premises and this information is not available to us. No significant deviation in either temperature control nor light cycles was reported.

Line 121: Reference not needed.

It seems interesting to us to cite a reference concerning the duration and age of puberty in rabbits, because these data are sometimes subject to approximation.

Lines 124-125: “At 12 weeks of age, rabbits were housed individually and received 150 g/d of diet”. (1) Provide information on the size of the individual cages. (2) No information concerning the size or the number of animals housed from 5 to 12 weeks is provided. Please add this information.

As the rabbits have been bred at Hypharm, in compliance with the European directive 98/58/EC on the protection of animals kept for farming purposes, transposed into French law by the ministerial order of 25 October 1982. The animals were housed in order to allow the breeder to control the food intake to respect the strategies chosen for the protocol.

Line 128: change “sacrificed” by “euthanized”. Describe the method used to euthanize the animals.

“euthanized by exsanguination” was added in the text

Lines 127-129: Split the sentence “At…fasting”. Suggestion: In the first phrase talk about the artificial insemination and the method used to declare pregnancy. In the second phrase precisely describe the moment and the method of euthanasia. An addition sentence explaining the reasons of the euthanasia is required. Example: The 40 pregnant females were euthanized at 14 weeks of gestation to obtain mammary gland tissues…

The paragraph has been modified accordingly to suggestions of the reviewers 1 and 2.

Line 129: “…hydrous fasting”. Not clear if feed or water was restricted. Please consider “…after 12 hours of fasting (water provided ad libitum)” Key question: How many animals you used in the study, from weaning (5 weeks) to 14 days of gestation to obtain the 40

pregnant females euthanized? Please indicate the total number of animals used, not only the 40 females euthanized. Mortality and infertility are normal outcomes in animal science.

An hydrous fasting consists of removing food while allowing *ad libitum* access to water in order to avoid any dehydration that could be responsible for the suffering of the rabbit and also for a disturbance of the blood metabolic parameters.

The breeder partner of the project, Hypharm, had committed to provide 40 rabbits in this protocol. We don't know if females were "taken" from the farm to be included in the research project or if they were produced specifically for this project. In this case, we have no information concerning mortality rate, quantity of rabbits produced for this protocol, or whether the surplus animals were integrated into other research protocols or the production chain.

Figure 1. Please change period names: - 5 to 9 weeks: fattening period ; - 9 to 11 weeks: pubertal period ; although it is mainly considered as part of the fattening period - 12 to 19 weeks: rearing period ; Key question: Why animals of SRAL group were limited to a daily ration of 150 g of feed per day from 12 weeks to 14 days of pregnancy instead of being fed ad libitum? Feeding restriction programs for young rabbit females may start as early as five weeks old (Rommers, 2004; https://doi.org/10.1051/rnd:2004037) or later (Martinez-Paredes et al., 2012; https://doi.org/10.1017/S1751731111002643), but in most cases (Pascual et al., 2013 ; https://doi.org/10.4995/wrs.2013.1236) feed restriction last for at least 9 weeks to have an effect on reproductive traits. Here, if I understand correctly, animals were subjected to four feed restriction plans during the fattening period (5 to 11 weeks; 11 weeks is the slaughter age in France), followed by a feed restriction plan of 150 g/days during the rearing period (12 to 19 weeks plus during the first 14 days of pregnancy). Please clarify this point in the introduction. To me, the confusion emanates from a lack of information on the periods of feed restriction in the studies quoted in the introduction (rearing periods for the Pascual et al., 2003 and Martinez-Paredes et al., 2019) and the period aimed here (fattening period).

Although the proposed terms are more in adequacy with the reality of the breeding, in a preoccupation with a better comprehension of the reader we kept the terms of "post-weaning" and "pubertal" which illustrate the physiological periods of sensitivity to the food.

This protocol was designed in partnership with the breeder and the company CCPA in order to measure the effects of restriction specially on the post-weaning and puberty, periods that we suspect/consider to be windows of susceptibility (Hue-Beauvais et al 2011 & 2019). Feeding females with 150g/d was defined by the breeders as non-restrictive, allowed us to observe the long-term effects (mid-gestation). *Ad libitum* feeding could caused a metabolic catch-up that would not have allowed us to measure the effects of the restriction on the mammary tissue, which could have been masked by a "high feeding" effect.

Lines 137-138: "then mammary samples were processed and stored for further analyses". Please describe the process used to conserve the mammary tissue and storage conditions.

According to the analyses, the conditions of conservation and storage of the samples are detailed in the paragraphs devoted to histological and RNA extraction techniques.

Line 151: Please move this information to the statistical analysis sub-section.

This information has been removed. The statistical section was fully modified to add additional analyses (ANOVA).

Line 175-179: Please provide information on the model used, as the estimation of the SEM depends on the model used. Provide also information on the distribution of the different variables studied. Are all variables normally distributed? For sample size, is it 30 samples per group? If yes, how do you achieve that as you have 10 samples per group (considering the animal as the statistical unit)? Differences should be declare only at a P-value bellow 0.05 and not bellow or equal to 0.05. Please correct it. Please indicate the statistical software used to perform the statistical analysis.

The statistical analyses has been revised, using ANOVA model. Sample size is 10 rabbits per group and significative difference is declared strictly lower than 0.05. The statistical software and packages used has been indicated in M&M section.

Lines 187-190: Consider exclude these sentences; no need to recall the experimental plan.

As suggested, the sentence has been removed.

Lines 190-193: This is the real restriction observed, following the restriction method proposed in the Material & Methods. Authors may present this information in the Material & Methods section, as this is the intended restriction rather than an observed result from the restriction applied. Key question concerning the calculation of feed intake during the feed restriction period (lines 193-194): How many animals per cage? How many cages? Females and male young rabbits where housed separately or together? There is no issue in presenting results of more animals (or statistical units: cages if group housing) than the 40 females used here to demonstrate the intake level of feed restriction. However, this information must figure in the manuscript. In addition, the real intake on the cages where the 40 females lived (number of cages, average and standard deviation for each group) must be presented as the “farm” average may not represent the real restriction the studied animals were subjected.

As we have been able to explain in advance, the rearing conditions were determined and measured in the case of the breeder who, depending on the stocking of the animals for the protocol, calculated the rations according to the formulae defined in the project (Fig 1B). These quantities were then administered to the animals individually by checking daily consumption. All the raw data from this project will be deposited on a sharing platform so that it can be consulted and shared.

Lines 194-196: Please indicate the value of the difference between the SR and MR groups. Provide the P-value for the difference observed at week seven. How many samples have been used in this calculation? Only the individual weight of the 40 rabbit does (10 per group) should be presented.

The statistical analyze has been revised and also the result paragraph concerning the effect of feeding strategies on body weight. The average body weight fro each group of 10 rabbit was presented.

Lines 197-199: Is a difference of +15.7 for the SRAL or +17.5 g/day for the MRAL groups respect to the restricted groups (SRR and SRR) significant (in terms of energy and nutrient ingested by the animals and statistically)? Present only the results concerning the 40 animals used in the study, not the farm average.

These data has been removed from the text to make the manuscript clearer.

Lines 200-203: provide the values of the differences between groups and the p-values of each comparisons.

All the significant P-Values are presented in the table of the figure 2B, revised accordingly with ANOVA analyze.

Figure 1 should be constructed only with the live weight of the studied 40 animals, not the farm average per restriction group (the error bars are too small for a sample of 10 individuals per group). Otherwise indicate in the material & methods that 40 females were sampled from four subpopulations of rabbits (n=X rabbits per group) that followed the following restriction plans… The results here presented appears to not reflect what is indicate in the material and methods section.

The figure 1 show the design and the description of the four feeding strategies applied. Food intake values were not shown. The number of rabbit per group was indicated in the M&M section.

Lines 203-206: Indicates the values of the differences in the text. Calculated only with the 10

At 12 weeks of age, group MRRAL animals show a higher body weight than group SRR rabbits (2795± 155g and 2421±99g respectively, $P<0.001$), and the same difference is observed between MRR and MRAL groups (2571±130g and 2795± 155g respectively, $P=0.047$)…. Data has been added.

Lines 210-212: comparison with the feed intake between SRR and MRR with SRAL and MRAL groups were not performed because of a lack of feed intake measurement for the restricted groups (140 g/day). Can you ascertain that every single cage in the 140 g/day restriction ended the daily feed allowance? Please indicates the standard deviation for each calculated mean (SRAL and MRAL). Only data on the 40 animals should be consider, see comments above.

The food intake data are not shown in the manuscript and data of average food has been removed from the figure to get the manuscript clearer.

Lines 213-217: Please revise this sentence to present the differences and P-values for a pairwise comparison between two groups at a time.

The sentence has been revised and the data with P values has been added.

Lines 225-228: Exclude the word “unexpectedly”. Start the sentence at “Unrestricted”. Present the only difference observed, between SRR and SRAL for cholesterol. Key question concerning the distribution of metabolic blood parameters measured: Indicate in the material and methods, section statistical analysis the distribution of these variables. Are they normally distributed?

Cholesterol levels (0.25±0.02 g/L for SRR group and 0.19±0.02 g/L for SRAL group , p=0.045 has been added in the text. Concerning the key question, metabolic variables are usually normally distributed around a defined average value depending on the parameters of the subject (species, age, physiological status, ...).

Lines 230-231: Do not mention the fertility rate, as you focus the samples on the 40 rabbit does being pregnant. Start at prolificacy and foetal viability. Correct Figure 4 A y-axis: “Average number of foetus per rabbit doe”.

The fertility rate has been removed in the text and the Y-axis of the figure 4 has been modified as suggested.

Lines 241-243: Exclude from this sentences the text: “*although … respectively).*” The alpha level chose to declare a statistical significance was set to <0.05. A value equal or above this value indicates a non-significance at the delta expected (*i.e.* the relevant difference between groups). To sustain your argument, a bigger sample size would be required to declare a difference of ~0.8 points of percentage (Figure 5 C).
Figure 5. Include a C letter for the graph presenting the “relative percentage of mammary tissue”

We are fully agree that a higher number of samples would hallowed us to obtain p-values inferior to the 5% threshold and that a value close to this threshold cannot be considered as significant, but nevertheless it can be an indicator of a trend when the whole dataset varies in a consistent way.
The B part of the represent the quantification of the mammary gland tissue and the duct lumen is considered as part of this tissue. The histogram is separated from the other data only to gain clarity because the value scale of its representation is much lower than that of the other mammary tissues.

Line 252: exclude “*…, and these differences were strongly significant”*.

This sentence seems justified in view of the comparisons between the different groups which show significant results with p values lower than 0.05.

Figure 6: Include the letters “A” and “B” for the left and right graphics, respectively.

The horizontal axis specifies the name of the gene whose expression is analyzed. Quoting the graphs using FasN or Scd respectively seems clearer to us than coding these graphs by A and B.

Lines 263-264: please correct this sentence. The effect is not related to the diet, it is related to the feeding strategy.
The text has been modified accordingly.

Figure 7: same comment as for Figure 6. Add here the letter “C”.

As for figure 6, hhe horizontal axis specifies the name of the milk protein gene whose expression is analyzed. Quoting the graphs using k casein, Wap or Lalba respectively seems us clearer than coding these graphs by A, B and C.

DISCUSSION

Line 286-287: please consider excluding *“although …performances.”*
This part of the sentence has bee removed in the manuscript.

Line 288: please change *“deciphered”* by *“studied”*. Exclude *“fertility”* (this was not studied – results not shown; lines 230-231).
Fertility was remover and replaced by “number and viability of fetuses”.

Lines 292-293: please explain the use of the word “***sensitivity***”. Do you mean, their daily energy and other nutrient requirements for growth are high, making the intake of animals in the MR group as high as 120.75 g/day (+25 g/d compared SR).
We use the word “sensitivity” to bring the concept that growing rabbit physiology could easily be upset by some slight modifications of nutrition during this period of development. This was illustrated by the fact that an exclusively quantitative difference of 25g/d induced a rapid and significant change in body weight during this intense growth phase.

Lines 293-298: Which effects? Please describe.
In our work we mainly consider the increase of the whole body mass. Tumova *et al* also described a decreasing body weight following short restrictive feeding period, but also more specific parameters such as gastrointestinal morphology and function.

Lines 298-299: These results underline that feed restriction was effective in limiting feed intake and growth. There is no direct relation between early-life being a critical period for nutrition. Which is important is to consider that feed restriction alter the normal development of the animal by delaying it. Please revise this sentence.
The sentence has been revised as well : These results underline the importance of considering young life, including post-weaning and puberty, as a critical period for nutrition and where feed restriction could alter the normal development.

Lines 299-301: Discuss the "harmonization" from the two angles treated. For the restricted group (140 g/day) animals gain weight, while for the *ad libitum* group the animals started a mild restriction (155.7 or 157.5 g/day to 150 g/day), but adapted quickly.
The results of the additional ANOVA statistical analysis showed no differences between the 4 groups during the fattening period, which is an argument in favor of weight harmonization, or the smaller groups were able to compensate for the weight gain.

Lines 301-303: In your case, growth does not seem to be irregular; no loss of live weight observed on the average curves (Figure 2 B). It is important to mention the negative consequence observed by Neave et al. (2019, dairy cows) and Haschke et al. (2019; humans) when a malnutrition event occur early in life. The present results does not evidence a long-term effect of a feed restriction plan on the measured parameters influencing the reproductive capacity nor a correct development of the mammary tissue. Does the increment on the adipose and epithelial tissues, followed by a reduction in the connective tissue affect the future nursing capacity of the rabbit females in the SRR group respect to the less restrictive feeding strategies?
We couldn't extrapolate further lactation capacity of the SRR group given the changes in development observed at mid-gestation. Although many studies, including our own, illustrate that modifications in mammary structure, observed at pregnancy (G8 or G14 in rabbit studies) are not favorable for subsequent lactations, we couldn't exclude a rescue of the phenotype. In most mammals, Dohad's concept supports the (more or less, depending on organ or function) harmful long-term consequences, often on future reproductive cycles, of nutritional deregulation before adulthood.

Lines 304-310: Please introduce in this part of your discussion the notion of the 'level' of the feed restriction (nutrient restriction) early in life, and its duration needed to affect the reproductive performance in the first reproductive event. In the present study, and with the different restriction plans adopted, can you conclude that more severe feeding strategy (SRR) impaired? Should a rabbit breeder using the SRR system be concerned?

Numerous studies, in many species (review Hue-Beauvais 2021, or Keady 2017 in ewes or Curtis 2018 in cattle,…), have shown different types of restriction, qualitative or quantitative, and their effect on reproductive capacities of females. Here, the restrictions applied are strategies that currently exist in breeding. It is therefore logical that even if they are not favorable to reproduction, they do not dramatically alter reproduction in the broad sense. However, question remains, whether it is favorable and physiologically sustainable to apply the feeding strategy to future female breeders as to meat-producing animals.

Line 312: A through revision on the lipid metabolism of restricted mammals is required. For instance, Yang *et al.* (2010; https://doi.org/10.1016/j.rvsc.2010.04.003) observed a lower body weight and a high serum total cholesterol in restricted fed broilers compared to *ad libitum* ones.

The sentences concerning the analyze of lipid metabolism has been revised and reference suggested concerning effect of early restriction on cholesterol level on poultry has been added.

Lines 313-316: No assessment on the stress was performed in the present study. To avoid speculation, please exclude this sentence.
Of course there is no stress induction in this protocol that can be directly related to the results obtained, nevertheless it is not possible to ignore this variable in ring protocols. As the groups had different treatments, we felt it was necessary to cite the works that link stress and metabolism, even if it would have been interesting to measure this parameter in our study.

Lines 319-322: Please provide a reference for the age of development of the mammary tissue in rabbit. The references provided here (Denamur, 1963 & Borellini, 1989) are not listed in the reference section. Please correct this issue. Authors should consider citing their own paper (Hue-Beauvais, 2019; https://doi.org/10.1002/dvdy.91).

The list of reference has been corrected and Hue-Beauvais 2019 has been added in the text.

Lines 334-336 and Lines 339-340: The results does not support this sentence. No statistical differences were detected.

Statistical analysis of this difference gives a P value of 0.05, which is at the limit of significance. It seemed interesting to us to mention this effect, which would probably be significant with a larger number of females. In the spirit of knowledge sharing, which incites us to file this article in a peer-community process, this seemed to us to be an interesting data to mention to the community.

Line 368: only discuss statistically significant results.
We discuss the results of expression of the Lalba gene, which shows significant differences, but the overall synthesis of milk proteins shows a similar effect. This data seems to us relevant to mention in the discussion and not in the results. Indeed, a global variation in the milk protein expression, even if some expression level are not significatively different, suggests a global effect of feeding strategies on mammary function, contrary to variation of a single milk protein gene which would suggest a more specific regulation effect leading to a modification in the milk composition.

Line 369: change "*(SR group vs MR group)*" by "*(SRAL vs MRAL groups)*".
Manuscript has been modified.

Line 386: change "*count*" by "*cost*".
Manuscript has been modified.

Line 396: Is it due to voluntary or involuntary culling? Please precise the reasons. The cause-consequence link is not direct.

Involuntary culling if the restriction strategy in early life induces a change in development (metabolism, mammary gland, etc.) which could alter reproductive performance and longevity, such as premature death of does after a few reproductive cycles (Lopes 2017 communication JRC).

Reviewer 3 : anonymous reviewer, 25 Mar 2022 09:42

The figure 1A is very helpful to understand the experimental plan, on the contrary the figure 2A is confounding,
for graphics, choose a style that can be clear even if you print in black and white

The legend of figure 2 has been modified to make the figure less confusing. The style was chosen to allow a perceptible contrast in grayscale and only the significant P-values were kept to facilitate reading.

line 285 what do you mean with “restrictive feeding strategies”?

The four feeding plan applied in this study are some strategies used by rabbit breeders and a based on different feed restriction rates to avoid weaning diarrhea.

MEC= I suggest avoiding this kind of abbreviation.

MEC for “Mammary Epithelila Cell” is a common abbreviation to avoid the heaviness of repetition inmammary development articles.

Line 324 ()

Removed in the text

I have some doubt about the utility and interpretation of gene expression related to synthesis of milk component in not lactating animals.

Synthesis of milk proteins begins at the beginning of gestation in mammals. The level of activation and expression of these genes are valuable molecular tools for estimating the state of differentiation of epithelial cells and mammary development (Sharp JA, 2016. doi: 10.1007/s10142-016-0485-0).