

1 **Impact of pre-breeding feeding practices on rabbit mammary gland development at**  
2 **mid-pregnancy.**

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## ABSTRACT

Optimizing rabbit does preparation during early life to improve reproductive performance is a major challenge for breeders. Does selected for reproduction have nutritional needs, which may not be supplied with the common practice of feed restriction during rearing in commercial rabbit production. Nutrition during early life was already known to influence metabolism, reproduction and mammary gland development later in life, in particular during pregnancy. The aim of this study was to analyze four different restriction feeding strategies during post-weaning and over the pubertal periods (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).

Unlike food intake, which remains regular, mean body weight gain was inversely proportional to the dietary restriction applied over the considered periods. The feeding strategies in place for the four groups have no effect on the reproductive parameters of the females, as opposed to certain metabolic parameters such as cholesterolemia, that vary with dietary intake. Furthermore, restriction programs have impacted mammary tissular structures at mid-pregnancy. The expression of lipid metabolism enzymes (Fatty acid synthase N and Stearoyl co-A desaturase) is also modified in mammary epithelial cells by the dietary strategies implemented. Moreover, milk gene expression, used as differentiation markers, indicates a better mammary epithelial development regarding further lactation, in the case of the less restrictive strategies during early life period, especially the higher feeding allowance.

Our results highlight the importance of investigating feeding conditions of young female rabbits and nutrition in early life rearing, in order to provide specific recommendations for optimizing lactation and thus preventing neonatal mortality of the offspring.

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## INTRODUCTION

54 Currently, in conventional rabbit farming in France, the cost of food represents 50% of the  
55 selling price of a rabbit. As a result, for the past 15 years, most rabbit breeders have  
56 implemented a feeding plan for growing rabbits (Gidenne, Combes, and Fortun-Lamothe 2012).  
57 These strategies had the advantage of reducing the risk of post-weaning digestive disorders and  
58 improving feed efficiency in the animals (Gidenne *et al.* 2009), while for the breeder, they  
59 presented both economic and environmental impacts (Zened *et al.* 2013).

60 In most breeding farms, post weaning (after 5 weeks of age) does are reared the same way as  
61 fattening animals (up to 10 weeks of age). Between 10 weeks of age and the first artificial  
62 insemination (AI) (19.5 weeks of age), young females receive a control quantity of feed daily.  
63 Nevertheless, feeding restriction frequently causes energy deficit leading to poor fertility  
64 (Pascual *et al.* 2003). Inadequate energy intake also impairs lactation and thus kits survival,  
65 growth and dietary transition, due to the level and quality of milk production (Martinez-Paredes  
66 *et al.* 2019).

67 Mammary gland is a complex secretory organ containing different tissues, one of the most  
68 important being mammary epithelial tissue, composed of different cell types, responsible for  
69 the synthesis and secretion of milk components. The growth and differentiation of mammary  
70 gland are lengthy processes that occur during early life and adulthood, including reproductive  
71 cycles (Macias and Hinck 2012). Factors that may influence mammary development during  
72 early life, can alter mammary development later at pregnancy and thus, impact the epithelial  
73 cell population, responsible for the synthesis and secretion of milk components during lactation  
74 (Robinson GW 1995).

75 Nutrition influences mammary gland development, with an impact depending on critical  
76 periods of susceptibility, such as weaning, puberty, pregnancy or lactation. In species, such as  
77 rabbit, administration of an obesogenic diet, from the neonatal period or during puberty induces  
78 deleterious effects on metabolism and mammary gland development later in life (Olson *et al.*

79 2010; Hue-Beauvais *et al.* 2019). Rearing does with fibrous diets increased the ability of  
80 primiparous females to obtain resources, especially at the onset of lactation (Martinez-Paredes  
81 2019).

82 Concerning the feeding strategies applied in breeding, studies showed detrimental effects on  
83 mammary gland development and metabolism induced by restriction followed by over  
84 allowance diet in gilts breeding (Farmer, Palin, and Martel-Kennes 2012). In the same way,  
85 feeding restriction was associated with modification of mammary epithelial tissue and milk  
86 properties in cattle farming (Stumpf *et al.* 2013). Finally, the increase of the feeding level during  
87 the post-weaning rearing period could be an interesting way in goat breeding, to enhance body  
88 development without impairing mammary gland development whilst having a positive impact  
89 on reproductive parameters such as litter weight (Panzuti *et al.* 2019) .

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91 The effect of restricted feeding management over early stages of life, such as post-weaning and  
92 puberty on mammary gland development during pregnancy and subsequent lactation remains  
93 still unknown. In this study, we investigate the impact of four feed restriction strategies used in  
94 female rabbits breeding, over two distinct periods (post-weaning and puberty) on different  
95 physiological aspects such as growth, metabolic profiles, reproductive parameters and  
96 mammary gland development on day 14 of first pregnancy corresponding to the transition from  
97 the proliferative phase of the mammary cells to the differentiation of the lobulo-alveolar  
98 structures to form acini (Lu and Anderson 1973).

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## MATERIALS AND METHODS

### 107 **Animals, experimental design and sampling**

108 This study was carried out in compliance with the French regulations on animal  
109 experimentation and with the authorization of the French Ministry of Agriculture. Protocol was  
110 approved by an Ethics Committee registered within the French Comité National de Réflexion  
111 Ethique sur l'Expérimentation Animale.

112 Forty female rabbits (Hyplus PS19) were housed individually in an indoor facility under  
113 controlled conditions of temperature (18°C) and light (usually an 8/16 h light/darkness cycle  
114 except for an inverted cycle during the week before mating). All rabbits received the same  
115 conventional commercial breeding diet (2,350 kcal of digestible energy, 15.2 % of crude  
116 protein, 17 % of celluloses, 0.56 % of digestible lysin). At weaning (5 weeks of age), the  
117 females were divided into two equivalent groups of 20 females, according to the body weight  
118 ( $862 \pm 61$  g). During the post-weaning period, from the fifth to the eighth week inclusive, the  
119 rabbits were weekly weighted and fed either with a strict restricted (SR), or a moderate  
120 restricted (MR) quantity of feed (Fig.1). During the pubertal period, for 3 weeks from 9 to 12  
121 weeks of age included (Hulot, Mariana, and Lebas 1982), both groups were randomly divided  
122 to form four experimental groups of 10 females, which received diet at 140 g/d (groups SRR:  
123 SR-Restricted and MRR: MR-Restricted ) or *ad libitum* (groups SRAL: SR-*Ad Libitum* and  
124 MRAL: MR-*Ad Libitum*) (Fig.1). At 12 weeks of age, rabbits were housed individually and  
125 received 150 g/d of diet. Then growth was determined by weighing the rabbits once a week to  
126 the age of 19 weeks. Food intake was also monitored on the same weekly basis during pubertal  
127 period for SRAL and MRAL groups. At 19 weeks of age, the females were mated by AI and  
128 then sacrificed on Day 14 of pregnancy, confirmed before euthanasia by abdominal palpation,  
129 after 12 hours of hydrous fasting.

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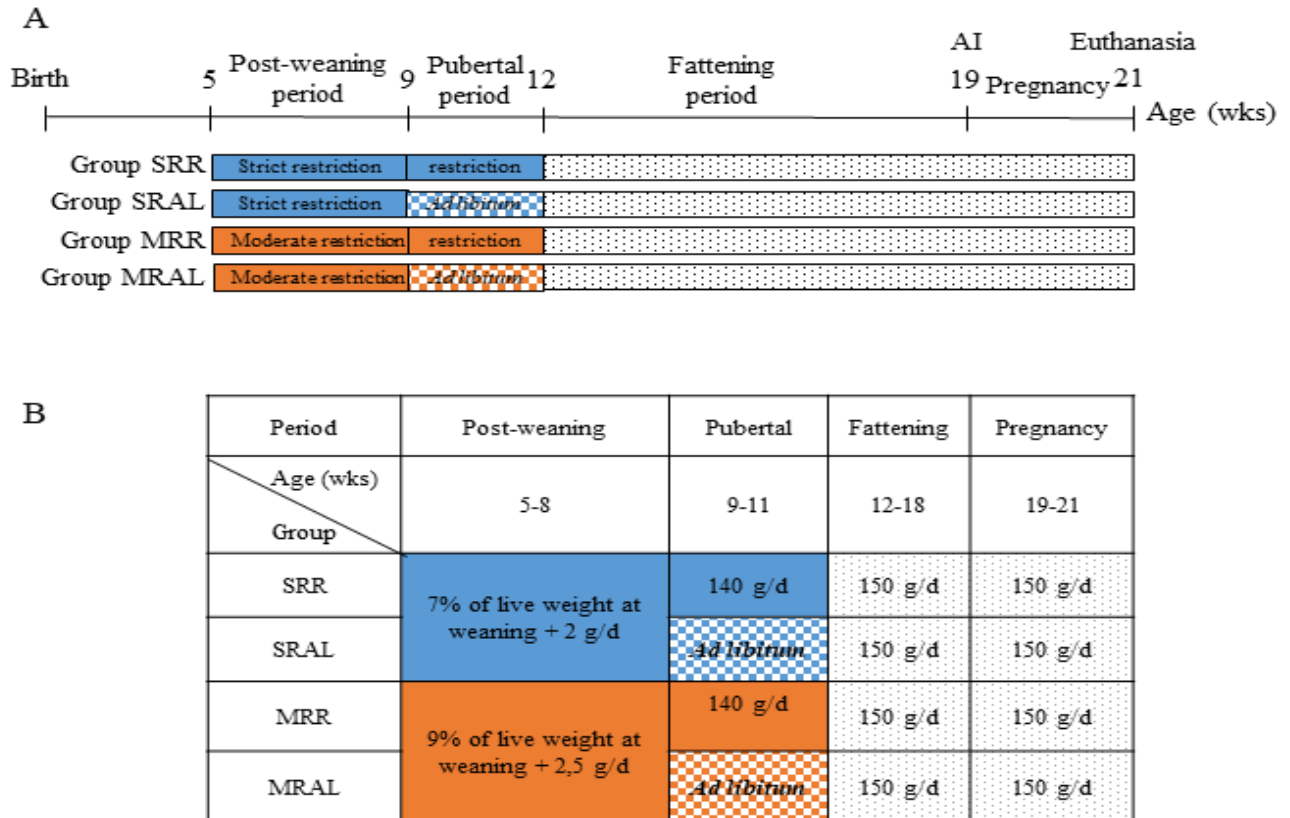


Figure 1 : (A) Design of the experimental protocol. Each group (n=10) received various quantities of food, over both post-weaning and pubertal periods . (B) Description of feeding strategies for each group : SRR : strictly restricted during post-weaning period and restricted during puberty; SRAL : strictly restricted during post-weaning period and fed *Ad libitum* during puberty; MRR : moderately restricted during post-weaning period and restricted during puberty; MRAL : moderately restricted during post-weaning period and fed *Ad libitum* during puberty;

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### 132 Sampling and metabolic assays

133 Blood samples of each forty females were collected, at euthanasia by exsanguination, in tubes  
 134 containing EDTA to determine levels of triglyceride (Triglyceride EnzyChrom kit;  
 135 Cliniscience), cholesterol (Cholesterol RTU kit; Biomerieux), glucose (Glucose RTU kit;  
 136 Biomerieux) and leptin (Cloud Clone Corp.). Left inguinal mammary gland from each 40  
 137 animal was fully excised and dissected to remove muscle, then mammary samples were  
 138 processed and stored for further analyzes. All embryo vesicles were dissected, opened and the  
 139 fetuses numbered and extracted to confirm viability by macroscopic examination.

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## 142 **Histological analysis**

143 For histology, samples were fixed for 24 hours in RC12 buffer (Alphelys, France) before  
144 embedding in paraffin. Five-micrometer sections, separated at least by 100  $\mu\text{m}$  each in the  
145 thickness of the tissue were mounted on slides. Slides were stained with hematoxylin and eosin  
146 (H&E; Sigma-Aldrich) and then digitized under bright light using a Hamamatsu NanoZoomer  
147 (Hamamatsu Photonics). Four sections per sample, i.e. eight sections per rabbit were processed  
148 and areas occupied by mammary epithelial tissue (clusters of alveolar structures), adipose  
149 tissue, mammary duct lumens or connective tissue were measured using CaseViewer software  
150 (3D Histech) and divided by the whole section area to generate the proportion of each tissue.  
151 Results are expressed as means  $\pm$  SEM.

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## 153 **RNA Extraction and RT-qPCR analyses**

154 Total RNA from mammary epithelial tissue was isolated from each mammary sample using the  
155 RNA NOW kit (Ozyme) according to the manufacturer's protocol. The integrity of ribonucleic  
156 acid was assessed using an Agilent Bioanalyzer. Samples with an RNA Integrity Number (RIN)  
157 higher than 7 were subsequently used (Fleige and Pfaffl 2006).

158 For quantitative PCR (qPCR) assays, reverse transcription (RT) was performed on 200 ng of  
159 each mammary sample's total RNA using the SuperScript VILO cDNA Synthesis kit according  
160 to the manufacturer's instructions (Invitrogen) and under the following conditions: 42°C for 60  
161 min and 85°C for 5 min. qPCR runs were achieved using Applied Biosystems SYBR Green  
162 PCR Mastermix (Thermo Scientific) according to the manufacturer's instructions, on a  
163 QuantStudio system (Thermo Scientific). After optimization of the qPCR systems (efficiency  
164 ranging from -3.25 to -3.45), amplification reactions were run in triplicate under the following  
165 conditions: 95°C for 15 min, 45 cycles of 95°C for 15 sec and 60°C for 1 min. The threshold  
166 cycles obtained for each gene were normalized with the values of the *TATA Binding Protein*  
167 (*Tbp*) gene and the results were expressed as fold changes of the threshold cycle (Ct) values

168 relative to the control using the 2- $\Delta\Delta$ Ct method (Livak and Schmittgen 2001). The primers used  
169 for each gene ( *$\kappa$ -casein*, *Whey acidic protein*,  *$\alpha$ -lactalbumin*, *Fatty acid synthase N* and *Stearoyl*  
170 *co-A desaturase*) amplified are presented in Table 1.

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172 Table 1 : Primer sequences used for qPCR experiments

Genes	Primer	Sequence 5'→3'
<i>Casein kappa</i>	Forward	GGAACAGACAACGTGCCGTG
	Reverse	CGAACCCAGCTACTACCTGC
<i>Whey acidic protein (Wap)</i>	Forward	T GCGCTATCTGGAACCCATC
	Reverse	GAGAGTTGGGCCTGAGTTCC
<i>Alpha-lactalbumin (Lalba)</i>	Forward	AT CAGCGATAAGCTGTGGTGT
	Reverse	ATTG ACCACTGGTTGGCACAT
<i>Fatty acid synthase N (FasN)</i>	Forward	ACCTCGTGAAGGCTGTGACTCA
	Reverse	TGAGTCGAGGCCAAGGTCTGAA
<i>Stearoyl-coA desaturase (Scd)</i>	Forward	TTATTCCGTTATGCCCTTGG
	Reverse	TTGTCATAAGGGCGGTATCC
	Reverse	GGTCTCTGCTGGCTTGTTC
<i>TATA Binding protein (Tbp)</i>	Forward	TGACCCCATGACCCCTATT
	Reverse	CAGCAAACCGCTTGGGATTA

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## 175 **Statistical analyses**

176 Experimental data are presented as means $\pm$ SEM (standard error of the mean). Statistical  
177 analyses were performed to detect significant inter-group differences using either unpaired  
178 Student's *t*-test when the sample size was >30, or the Mann-Whitney U-test when the sample  
179 size was <30).  $P \leq 0.05$  was considered to be significantly different.

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## RESULTS

187 In this study, the impact of feeding strategies encountered in commercial breeders, where does  
188 are often reared the same way as the fattening animals, with moderate to severe feed restriction,  
189 has been tested. Different levels of restriction during 2 separate periods, covering the early-life  
190 from 5 to 12 weeks of age have been compared (Fig. 1). During the first period of restriction  
191 which covers the post-weaning period and lasts for 4 weeks, one group (SR group) received a  
192 more drastic restriction than the second group (MR group) (mean quantity of feed: SR group  
193 95 g/d and MR group 120,75 g/d). At the beginning of the post-weaning period, mean body  
194 weight of does was  $832\pm 61$  g, equally distributed among SR and MR groups. During the first  
195 two weeks, from 5 to 6 weeks of age, body weight was similar between group SR and MR (Fig  
196 2), but from 7 weeks of age, does of group MR weighted higher than those of group SR.  
197 At 9 weeks of age, each group was divided into 2 sub-groups according to the quantity of food  
198 received, restriction feeding with 140 g/d (groups SRR and MRR) or *ad libitum* (groups SRAL  
199 and MRAL, mean quantity of feed: group SRAL 155,7 g/d and group MRAL 157,5 g/d).  
200 The difference of weight observed during the late post-weaning period remained significant  
201 during the pubertal and fattening periods and depends on the restriction feeding pattern. At the  
202 end of the pubertal period, from 9 to 11 weeks of age, group SRR rabbits have the lowest weight  
203 curve. At 12 weeks of age, group SRAL animals show a higher body weight than group SRR  
204 rabbits, and the same difference is observed between MRR and MRAL groups: rabbits strongly  
205 restricted in post weaning and fed *ad libitum* during puberty (group SRAL), reached the same  
206 weight than rabbits less restricted in post-weaning and restricted during pubertal (group MRR).  
207 Furthermore, whatever the diet during the post-weaning period, animals fed *ad libitum* during  
208 the puberty period are heavier than those fed restricted diets (SRAL mean weight higher than  
209 SRR and MRAL mean weight higher than MRR, Fig 2B). As the groups SRAL and MRAL  
210 were fed *ad libitum*, the mean food intake was calculated during the pubertal period. No

211 significant difference was observed between both groups (155.7 g/d for SRAL and 157.5 g/d  
212 for MRAL) (Fig 2A).

213 At the beginning of the fattening period (12 weeks of age), the weight of the does was higher  
214 in the less restricted group (MRAL) than in the SRAL and MRR groups (2795.6±55.1 g for  
215 MRAL, 2597.0±49.3 g for SRAL and 2571±49.3 g for MRR,  $p<0.05$ ) which were similar to  
216 each other, and in the SRR group in which the mean weight was the lowest (2423.3±37.6 g,  
217  $p>0.05$ ). From the 13<sup>th</sup> week of age until the end of the fattening period (18<sup>th</sup> week of age)  
218 during which all the rabbits were receiving the same quantity of feed, only body weights of  
219 groups SRAL vs. MRAL were different at weeks 14 and 15. At the end of the fattening period,  
220 body weights were equivalent within the four groups (Fig 2B).

A

Age (wks)	Group SR		Group MR	
5-8	95 g		120,75 g	
	Group SRR	Group SRAL	Group MRR	Group MRAL
9-11	140 g	155,7* g (ad libitum)	140 g	157,5* g (ad libitum)
12-18	150 g	150 g	150 g	150 g

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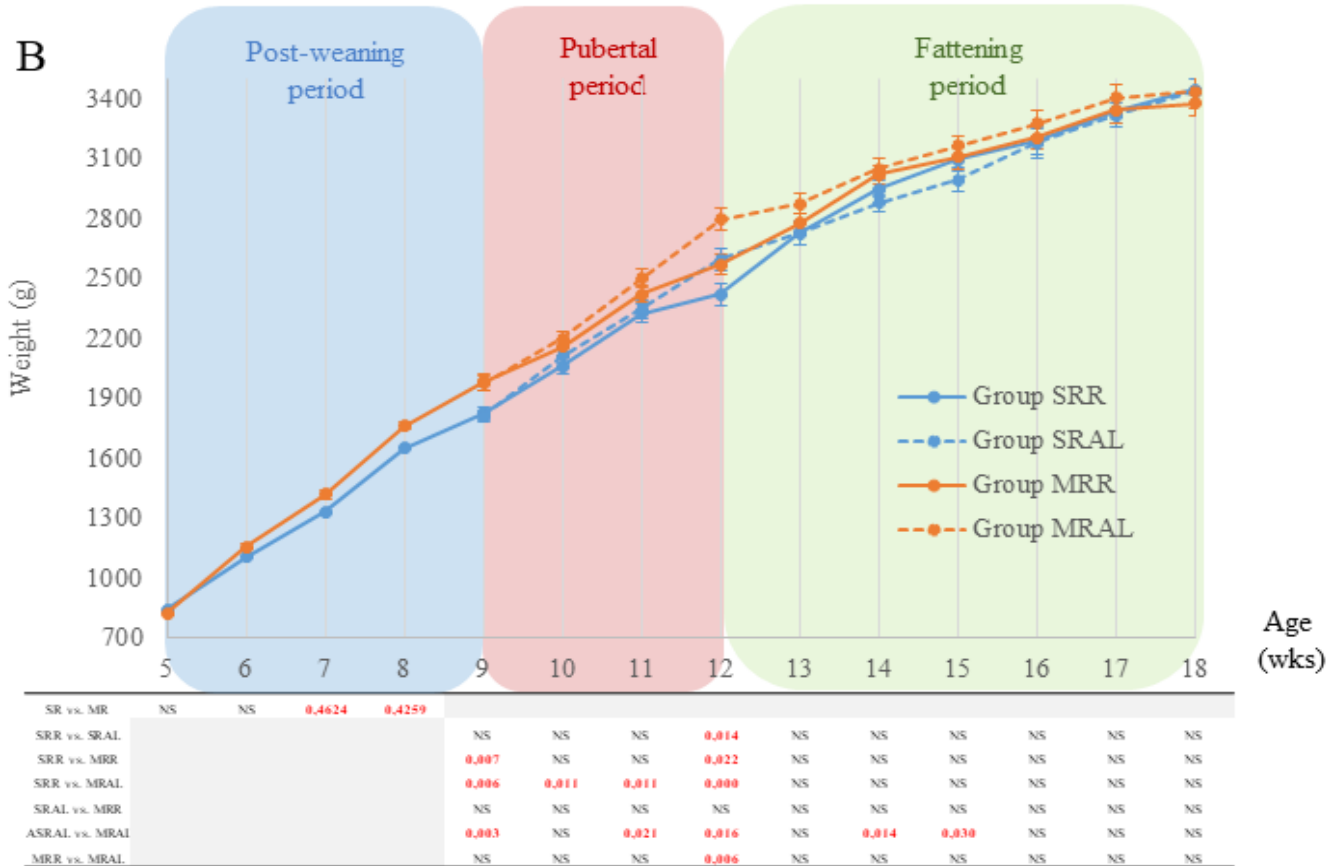


Figure 2: Effect of the four feeding strategies over three periods of age, in female rabbits.

(A) Average daily food intake. Data are expressed by Mean  $\pm$  sem. (B) Body weight, weekly measured in the four different groups of female rabbits, between 5 and 18 weeks of age. \*Data obtained by measuring ingestion. Data are expressed by Mean  $\pm$  sem. Significant differences ( $P < .05$ ) between the groups are indicated below. NS means Non Significant.

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222 Metabolic profiles were evaluated at mid-pregnancy by measuring glucose, cholesterol,

223 triglycerides and leptin in blood after 12 hours of fasting (Fig 3). No difference was observed

224 between the four feeding strategies concerning glycemia, triglyceridemia or leptinemia at mid-

225 pregnancy. Unexpectedly, unrestricted feeding during pubertal period when animals received a

226 restricted feed during post-weaning period (groups SRR vs. SRAL) provoked a significant

227 decrease in cholesterol levels (Fig 3). This difference was not observed among does which were  
228 less restricted during the post-weaning period (groups MRR vs. MRAL).

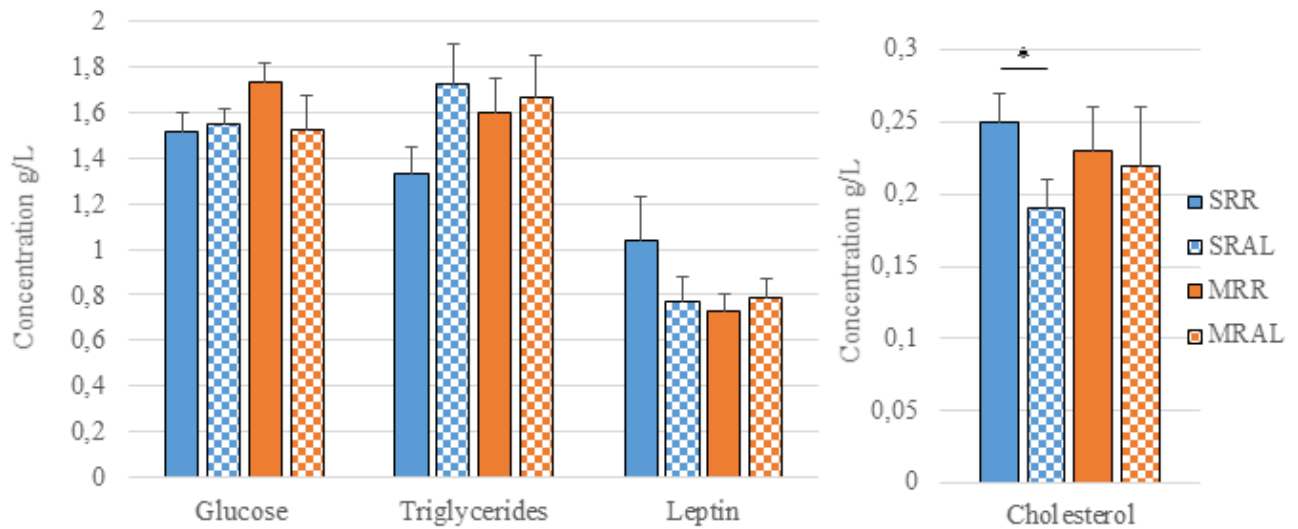


Figure 3: Metabolic profiles of each group (n=10) at mid-pregnancy. Data are expressed as means  $\pm$  SEM. Significant differences ( $p < 0.05$ ) between the groups are indicated by asterisks (\*).

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230 The feeding strategies had no effect on the reproductive parameters: the fertility rate (data not  
231 shown), prolificacy and the fetal viability (Fig 4) were not different in the four groups.

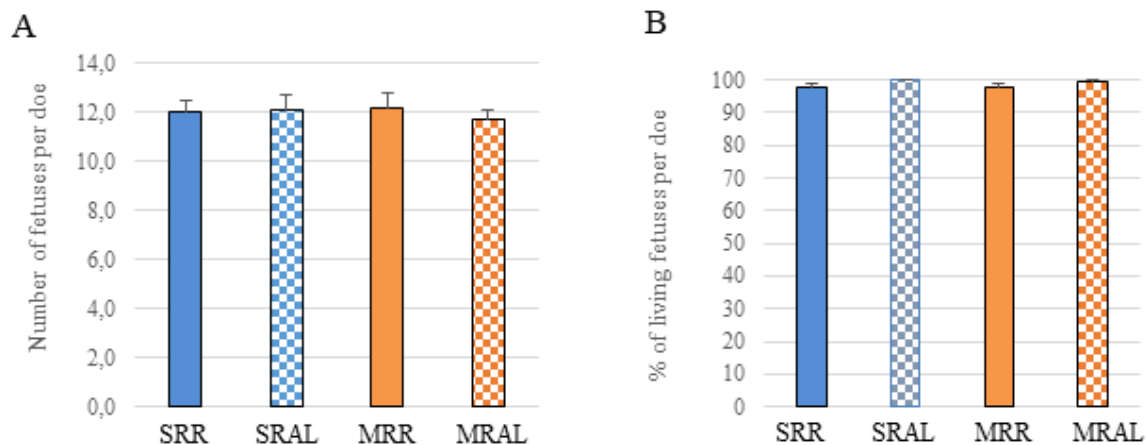


Figure 4 : (A) Average number of fetuses per doe in each group (n=10). (B) Average percentage of living fetuses per doe in each group.

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234 To examine the effects of the feeding strategies during both post-weaning and pubertal periods  
235 on mammary gland, histological analyses were performed. The examination of mammary tissue  
236 sections from all does on Day 14 of pregnancy revealed some differences between the four

237 groups (Fig 5). The surface occupied by mammary connective tissue increased, while adipose  
238 and epithelial tissues decreased in group SRAL compared to group SRR (Fig 5B). This  
239 difference was not observed when does received a less restricted diet during the post-weaning  
240 period (groups MRR vs. MRAL). The area corresponding to the ducts' lumina was not  
241 significantly different within the four groups. (Fig 5B), although a declining trend can be  
242 observed in the less-restricted groups during the post-weaning period (MRR and MRAL  
243 compared to SRR or SRAL groups;  $p=0.06$  and  $p=0.056$ , respectively).

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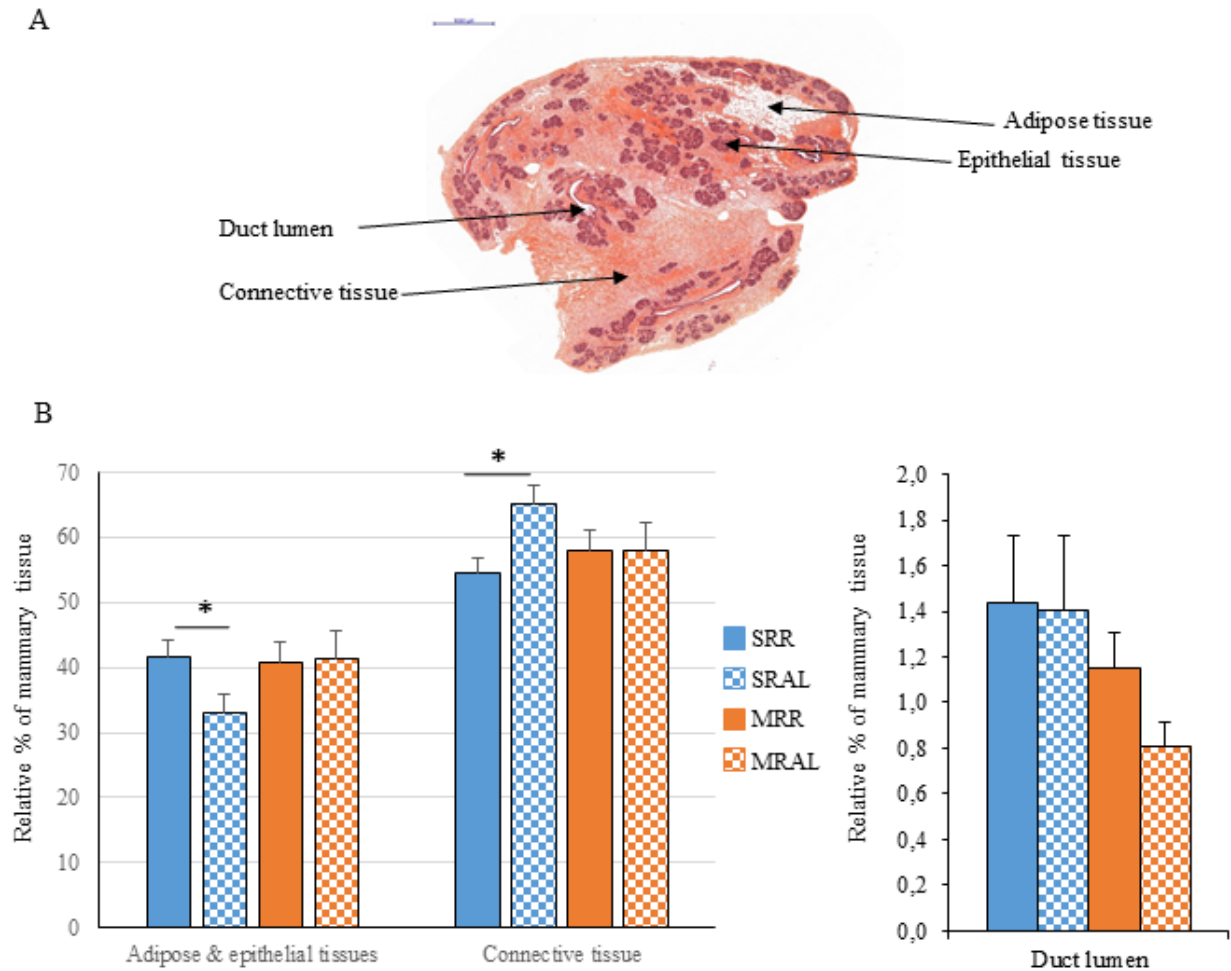


Figure 5: Histological analyses of mammary gland on Day 14 of pregnancy. (A) Representation of the different types of tissues present on a representative histological section of mammary gland, stained with hematoxylin and eosin. Scale bar represents 1mm (B) Relative quantification of mammary gland tissues: adipose and epithelial tissues, connective tissue, and duct lumen. Data are expressed as means  $\pm$  SEM. Significant differences ( $P < 0.05$ ) between the groups are indicated by asterisks (\*). Number of animals per group  $n = 10$ .

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246 In order to study the modifications induced by the different feeding strategies, mammary  
247 epithelial cell differentiation status, using mammary differentiation markers such as enzymes  
248 involved in lipid metabolism (*Fatty acid synthase N (FasN)* and *Stearoyl-coA desaturase (Scd)*)  
249 as well as milk proteins (*kappa casein*, *Whey acidic protein (Wap)* and *alpha-lactalbumin*  
250 (*Lalba*)) were assessed. *FasN* expression increased when less restricted feeding strategies were  
251 used (Fig 6). Indeed, higher levels of *FasN* transcripts were observed in groups MRR and  
252 MRAL than in groups SRR and SRAL, and these differences were strongly significant. *Scd*

253 transcript levels increased between groups SRR and SRAL and MRAL, but were similar in  
254 groups SRAL, MRR and MRAL (Fig 6).

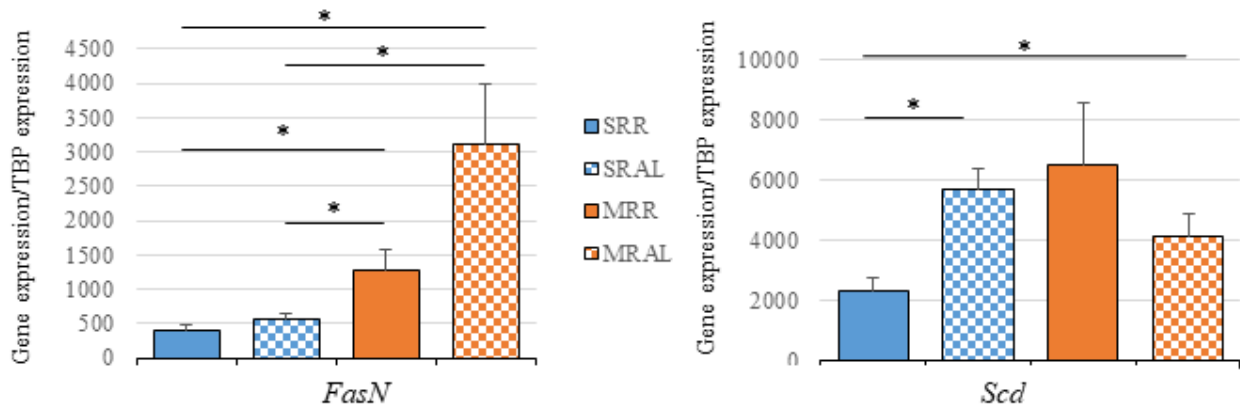


Figure 6: Expression of genes involved in lipid metabolism in mammary epithelial tissue on Day 14 of pregnancy. Analyses were performed in the four groups of rabbits (n=10 in each group) according to the feeding strategy (groups SRR to MRAL). The expression of transcripts was assessed for *Scd* and *FasN* and normalized with *Tbp* as housekeeping gene. Data are expressed as means  $\pm$  SEM. Significant differences (at least  $P < 0.05$ ) between the groups are indicated by asterisks (\*).

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256 To further investigate the changes induced by the different feeding strategies in mammary  
257 epithelial tissue, we analyzed the patterns of expression of genes encoding milk proteins (*kappa*  
258 *casein*, *Whey acidic protein (Wap)* and *alpha-lactalbumine (Lalba)*), using RT-qPCR (Fig. 7),  
259 since these genes are specific markers for differentiated mammary epithelial cells (MEC). In  
260 all groups a high individual variability within the MRR group was observed. However, analyses  
261 revealed no difference between groups concerning the *κ-casein* gene expression. *Wap*  
262 expression profile was similar between SRR and SRAL as well as MRR and MRAL groups,  
263 The *Lalba* transcript level was higher in MRAL than in SRAL group highlighting an effect of  
264 diet during post-weaning period on the expression of this gene when the animals are fed *ad*  
265 *libitum* during pubertal period.

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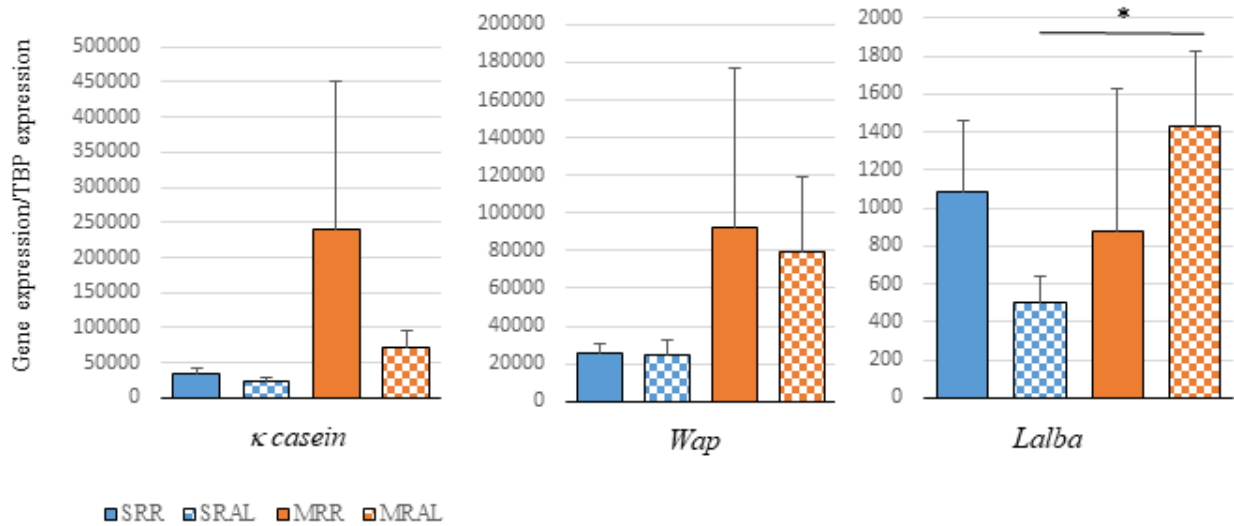


Figure 7:  $\kappa$  casein, Wap and Lalba expression in mammary epithelial tissue on day 14 of pregnancy of the four groups of rabbits (n=10 in each group), according to the feeding strategies (groups SRR to MRAL). Transcripts levels were assessed for  $\kappa$  casein, Wap and Lalba, and normalized with Tbp as housekeeping gene. Data are expressed as means  $\pm$  SEM. Significant differences (at least  $P < 0.05$ ) between the groups are indicated by asterisks (\*).

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## DISCUSSION

283 For economic and logistical reasons, does, whether they are intended for meat production or to  
284 become the future breeders, are often fed the same way in the farms. This means that the animals  
285 are subjected to restrictive feeding strategies during their early-life periods, such as post-  
286 weaning and puberty, although such diets may not be designed to optimize their reproductive  
287 and lactation performances. Here, influence of different combinations of dietary restrictions  
288 was deciphered, with a particular focus on metabolism, fertility and mammary gland  
289 development, in rabbits at mid-pregnancy.

290 Our results showed that restrictive feeding changes starting at the post-weaning period provoke  
291 a difference in body weight. This difference occurs from the third week of diet, and is due to a  
292 difference of approximately 25g/d in the quantity of feed, thus emphasizing the high sensitivity  
293 of growing rabbit to nutrition during the post-weaning period. Such effects have already been  
294 observed in rabbits submitted to a short intensive food restriction followed by a re-alimentation  
295 period (Tumova *et al.* 2016). Feeding strategies during pubertal period have shown that *ad*  
296 *libitum* groups (SRAL and MRAL) have increased food intakes (> 150 g/d) than restricted  
297 groups SRR and MRR, which received 140 g/d. Consequently, body weight is higher in the less  
298 restricted-fed groups (SRAL and MRAL) at the end of pubertal period. These results underline  
299 the importance of considering early-life as a critical period for nutrition. Switching during the  
300 fattening period to a quantitatively identical diet for all four groups harmonized the body weight  
301 of all the does. Moreover this is consistent with studies showing that irregular weight growth  
302 in response to feeding has long-term consequences in adulthood (Neave *et al.* 2019; Haschke  
303 *et al.* 2019).

304 Nevertheless, mechanisms involved still remain unclear in particular regarding the impact of  
305 feeding restriction on reproduction (Villeneuve, Cinq-Mars, and Lacasse 2010b). According to  
306 previous work, restricted feeding strategies tested did not impair reproductive performances at  
307 pregnancy, while it has been showed, in ovine that specific plan of food restriction could affect

308 the onset of puberty, and negatively influence the hypothalamic-pituitary-ovary axis involved  
309 in the hormonal control during pregnancy (Villeneuve, Cinq-Mars, and Lacasse 2010a; Rizzoto  
310 *et al.* 2019; Wang *et al.* 2016).

311 Measurements of blood metabolic parameters are commonly used to assess the effect of diets  
312 on body physiology. Surprisingly, analysis of metabolic parameters at mid-gestation in does  
313 showed a higher level of plasma cholesterol in the most restricted group. This finding could be  
314 also related to stress induced by restriction feeding since a relationship has been observed  
315 between plasma cholesterol and leptin concentrations and stress-induced disorders (Shankar *et*  
316 *al.* 2012; Jow, Yang, and Chen 2006). The variations in the applied restriction feeding strategies  
317 were only quantitative and weak. This may explain the subtle variations in the metabolic  
318 parameters measured in our study.

319 In early life, the mammary gland is a potent target for environment effects, particularly those  
320 related to nutrition, because the mammary epithelium has entered a stage of growth (Denamur  
321 1963) leading to the establishment of structures that will differentiate to produce milk  
322 components (Borellini F 1989). The first half of gestation is essentially dedicated to the  
323 proliferation of mammary epithelial cells (MEC), while the second half is rather characterized  
324 by the differentiation of these cells. At mid-pregnancy (Day 14), () interstitial adipose tissue  
325 gradually disappears and proliferating MEC fill in the inter-ductal spaces and start to express  
326 genes that can be considered as differentiation markers, such as milk protein genes (Robinson  
327 1995). This is a particularly opportune time to estimate and analyze the mammary consequences  
328 of nutritional changes that occurred in early life. Moreover, changes to mammary gland  
329 development during pregnancy can impact the MEC population that is responsible for the  
330 synthesis and secretion of milk components in lactation (Robinson 1995).

331 No drastic changes in mammary gland histology were observed according to the different  
332 feeding strategies, which is reassuring since these strategies are based on restrictions practiced  
333 in the farms. However, a significant increase in connective tissue was observed in group SRAL

334 compared to SRR, due to a combined decrease in fat and epithelial tissues. Within the mammary  
335 ducts, an increased luminal area in the most restricted animals during the post-weaning period  
336 compared to the less restricted animals was observed. In addition, the weaker ductal areas were  
337 measured in not restricted rabbits during puberty.

338 Our results suggest that a severe feeding restriction, during post-weaning period, followed by  
339 *ad libitum* feeding during puberty may have deleterious effects on the mammary gland  
340 development and disturb its tissular composition, as observed here in pregnancy.

341 Consistent results were found in gilts showing that the impact of a strong restriction followed  
342 by unregulated feeding had negative consequences, among others on mammary gland  
343 development (Farmer, Palin, and Martel-Kennes 2012; Farmer *et al.* 2004). This dietary  
344 dichotomy between these two life stages appears to be more deleterious than a restriction  
345 throughout the only post-weaning period. Indeed, in case of prolonged restriction, the body  
346 adapts and allows the preservation of mammary gland development (Park *et al.* 1994).

347 Lipid metabolism in mammary tissue was examined, using characteristic enzyme expression to  
348 correlate the defect in epithelial and adipose development with putative modifications in  
349 mammary function and differentiation. Fatty acid synthase N (FASN) and Stearoyl-coA  
350 desaturase (SCD) are both involved in the fatty acids biosynthesis and are found in cell types  
351 with a high lipid metabolism, such as MEC (Suburu *et al.* 2014). FASN is a rate-limited enzyme  
352 for fatty acid *de novo* biosynthesis in particular the long-chain fatty acids biosynthesis (Shi et  
353 al. 2015). *Fatty acid synthase N (FasN)* gene expression was increased in mammary glands of  
354 does with less restrictive nutritional status (SRR<SRAL<MRR<MRAL), which is consistent  
355 with the fact that intensive lipogenesis is correlated with higher level of nutrient intakes  
356 (Takeuchi *et al.* 2001).

357 Stearoyl-CoA desaturase (SCD) regulates membrane fluidity by the conversion of endogenous  
358 and exogenous saturated fatty acids into mono-unsaturated fatty acids (Angelucci *et al.* 2018).

359 Overall, *Scd* gene expression appeared to decrease with high dietary restriction. Interestingly,

360 increased levels of *Scd* transcripts were found in the group with the lowest cholesterol level  
361 (SRAL), thus contributing to correlate high SCD concentrations to metabolic disorders (Igal  
362 2011; Tsiplakou *et al.* 2015) as well as confirming the inversely proportional relation between  
363 cholesterol and SCD (Tian *et al.* 2018).

364 To determine whether feeding strategies during post-weaning and/or pubertal periods can affect  
365 MEC differentiation, expression of milk protein genes was performed. Kappa casein, WAP and  
366  $\alpha$ -Lactalbumin, are expressed exclusively in the MEC and can be used as MEC differentiation  
367 markers.

368 The three milk proteins' transcripts tend to be increased in the less restriction feeding groups  
369 during post-weaning period (SR group *vs.* MR group), suggesting MEC differentiation. Those  
370 results in milk gene expression might indicate a better commitment toward mammary epithelial  
371 tissue function and lactation with the less restrictive strategies, especially with higher feeding  
372 allowance in early life period.

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374 The impact of diet and nutritional strategies has been studied in different species, especially in  
375 farm animals. Among these, mammary gland development and the resulting milk production  
376 capacity has been related mainly to the qualitative aspect of the diet (hypo- or hypercaloric diet,  
377 low protein diet, etc.) (Bautista *et al.* 2013; Fernandez-Twinn *et al.* 2010; Hue-Beauvais *et al.*  
378 2011). The impact of moderate feeding restriction on mammary gland development remains a  
379 little studied subject, depends on the species considered, and on the fate of the animal whether  
380 it is raised for meat, milk production or reproductive capacity. In ovine, effects of restricted  
381 feeding before puberty did not shown any negative impacts on mammary gland development,  
382 reproduction, lactation and offspring growth performance (Villeneuve, Cinq-Mars, and Lacasse  
383 2010a, 2010b). In the case of pig farming, the balance between feeding and mammary gland  
384 development is much more delicate (Farmer 2018).

385 In rabbit breeding, feed restriction strategy is widespread used to improve productivity,  
386 decreased mortality and economic count. For fattening rabbits, feed restriction during rearing  
387 period allowed uniformity in body weight and decreased neonatal mortality (Rommers *et al.*  
388 2001). While a moderate feeding restriction may improve some sperm morphologic  
389 characteristics, as well as fertility in male rabbits (Pascual *et al.* 2016), it seems that restriction  
390 strategies have shown less beneficial effects and even more for rabbit does (Birolo *et al.* 2020).  
391 Restricting feeding during different stages of pregnancy, even if it does not strongly affect  
392 growth of young rabbits, may delay placental growth, decrease the offspring survival and birth  
393 weight (Rommers *et al.* 2004; Matsuoka *et al.* 2012; Manal, Tony, and Ezzo 2010). In early  
394 life, rearing does with the same feeding strategies as fattening rabbits, leads to energy deficit,  
395 body mobilization and may reduce reproductive performance (Fortun-Lamothe 2006).  
396 Consequently, feeding strategies and lifespan are closely linked in rabbit does.  
397 Our findings showed that feed restriction strategies applied during post-weaning and/or pubertal  
398 period can impact mammary gland structure and may delay mammary epithelial tissue  
399 development and functionality. These results also suggest the urgent need of further  
400 investigations on milk composition and subsequent lactation capacity of restricted does through  
401 several reproductive cycles to provide recommendations. Indeed, while a moderate feeding  
402 restriction does not necessarily have consequences, a severe one could adversely affect health  
403 and breeding performances.

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