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

Hue-Beauvais[1*] C, Bébin K[2], Robert R[3], Gardan-Salmon D[4], Maupin M[3], Brun N[1], Aujean E[1], Simon S[1], Charlier M[1] & Le Provost F[1]

[1]GABI, INRAE, AgroParisTech, Université Paris-Saclay, 78350, Jouy-en-Josas, France

[2]CCPA Group, Z.A du bois de Teillay, 35150, Janzé, France

[3] Hypharm SAS, 3 La Corbière, Roussay, 49450, Sèvremoine, France

[4] Deltavit, CCPA Group, Z.A du bois de Teillay, 35150, Janzé, France

*Corresponding author: cathy.hue-beauvais@inrae.fr Phone +33 1 34 65 25 50

Running head: Feeding strategies influence mammary development

Key words: Rabbit, mammary gland, feeding strategy, epithelial cells

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.

INTRODUCTION

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

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

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

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

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

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

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

105

106 # MATERIALS AND METHODS

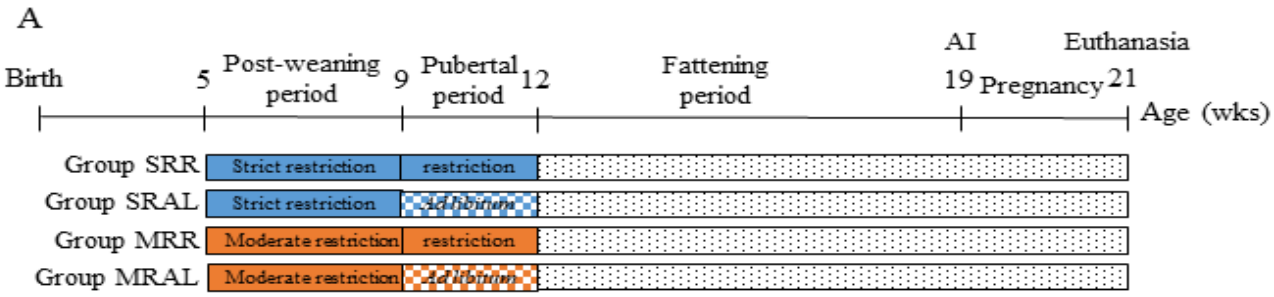
107 ## Animals, experimental design and sampling

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 ± 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.

130

A



B

Period	Post-weaning	Pubertal	Fattening	Pregnancy
Age (wks) / Group	5-8	9-11	12-18	19-21
SRR	7% of live weight at weaning + 2 g/d	140 g/d	150 g/d	150 g/d
SRAL		*Ad libitum*	150 g/d	150 g/d
MRR	9% of live weight at weaning + 2,5 g/d	140 g/d	150 g/d	150 g/d
MRAL		*Ad libitum*	150 g/d	150 g/d

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; SRR : moderately restricted during post-weaning period and restricted during puberty; MRAL : moderately restricted during post-weaning period and fed *Ad libitum* during puberty;

131

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.

140

141

Histological analysis

For histology, samples were fixed for 24 hours in RCl2 buffer (Alphelys, France) before embedding in paraffin. Five-micrometer sections, separated at least by 100 µm each in the thickness of the tissue were mounted on slides. Slides were stained with hematoxylin and eosin (H&E; Sigma-Aldrich) and then digitized under bright light using a Hamamatsu NanoZoomer (Hamamatsu Photonics). Four sections per sample, i.e. eight sections per rabbit were processed and areas occupied by mammary epithelial tissue (clusters of alveolar structures), adipose tissue, mammary duct lumens or connective tissue were measured using CaseViewer software (3D Histech) and divided by the whole section area to generate the proportion of each tissue. Results are expressed as means ± SEM.

RNA Extraction and RT-qPCR analyses

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

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

relative to the control using the 2-ΔΔCt method (Livak and Schmittgen 2001). The primers used for each gene (*κ-casein, Whey acidic protein, α-lactalbumin, Fatty acid synthase N and Stearoyl co-A desaturase*) amplified are presented in Table 1.

Table 1 : Primer sequences used for qPCR experiments

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

Statistical analyses

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

RESULTS

In this study, the impact of feeding strategies encountered in commercial breeders, where does are often reared the same way as the fattening animals, with moderate to severe feed restriction, has been tested. Different levels of restriction during 2 separate periods, covering the early-life from 5 to 12 weeks of age have been compared (Fig. 1). During the first period of restriction which covers the post-weaning period and lasts for 4 weeks, one group (SR group) received a more drastic restriction than the second group (MR group) (mean quantity of feed: SR group 95 g/d and MR group 120,75 g/d). At the beginning of the post-weaning period, mean body weight of does was 832±61 g, equally distributed among SR and MR groups. During the first two weeks, from 5 to 6 weeks of age, body weight was similar between group SR and MR (Fig 2), but from 7 weeks of age, does of group MR weighted higher than those of group SR.

At 9 weeks of age, each group was divided into 2 sub-groups according to the quantity of food received, restriction feeding with 140 g/d (groups SRR and MRR) or *ad libitum* (groups SRAL and MRAL, mean quantity of feed: group SRAL 155,7 g/d and group MRAL 157,5 g/d).

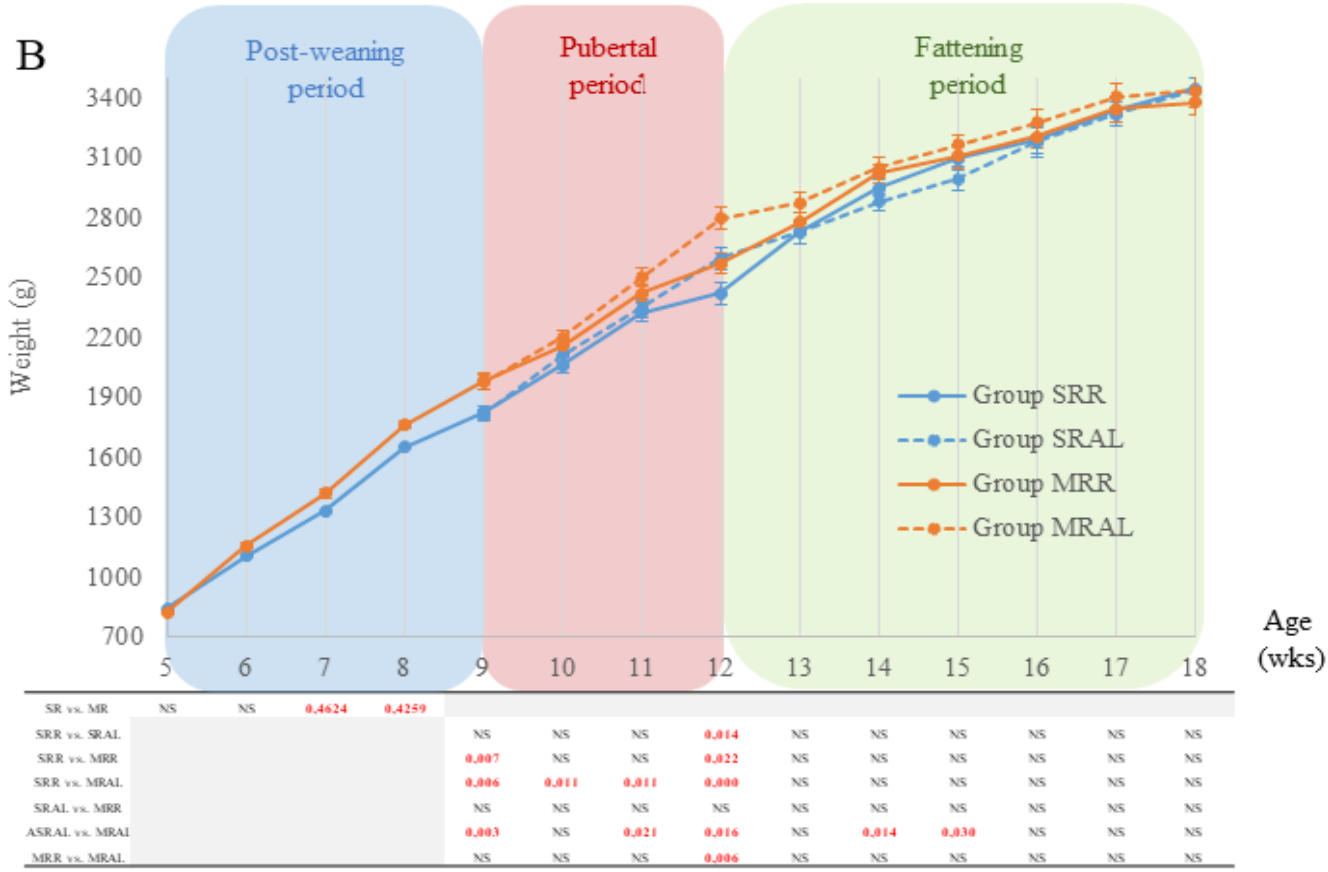
The difference of weight observed during the late post-weaning period remained significant during the pubertal and fattening periods and depends on the restriction feeding pattern. At the end of the pubertal period, from 9 to 11 weeks of age, group SRR rabbits have the lowest weight curve. At 12 weeks of age, group SRAL animals show a higher body weight than group SRR rabbits, and the same difference is observed between MRR and MRAL groups: rabbits strongly restricted in post weaning and fed *ad libitum* during puberty (group SRAL), reached the same weight than rabbits less restricted in post-weaning and restricted during pubertal (group MRR). Furthermore, whatever the diet during the post-weaning period, animals fed *ad libitum* during the puberty period are heavier than those fed restricted diets (SRAL mean weight higher than SRR and MRAL mean weight higher than MRR, Fig 2B). As the groups SRAL and MRAL 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 13th week of age until the end of the fattening period (18th 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

B



SR vs. MR	NS	NS	0,4624	0,4259								
SRR vs. SRAL					NS	NS	NS	0,014	NS	NS	NS	NS	NS	NS
SRR vs. MRR					0,007	NS	NS	0,022	NS	NS	NS	NS	NS	NS
SRR vs. MRAL					0,006	0,011	0,011	0,000	NS	NS	NS	NS	NS	NS
SRAL vs. MRR					NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
ASRAL vs. MRAL					0,003	NS	0,021	0,016	NS	0,014	0,030	NS	NS	NS
MRR vs. MRAL					NS	NS	NS	0,006	NS	NS	NS	NS	NS	NS

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 ± 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 ± sem. Significant differences ($P < .05$) between the groups are indicated bellow. NS means Non Significant.

221

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).

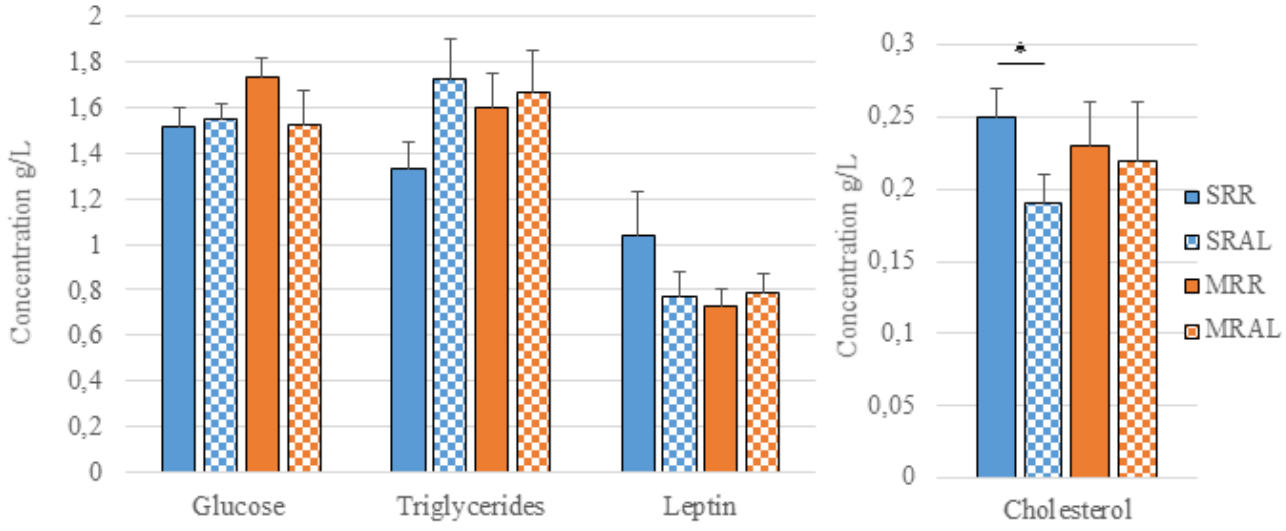


Figure 3: Metabolic profiles of each group (n=10) at mid-pregnancy. Data are expressed as means ± SEM. Significant differences ($p < 0.05$) between the groups are indicated by asterisks (*).

229

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.

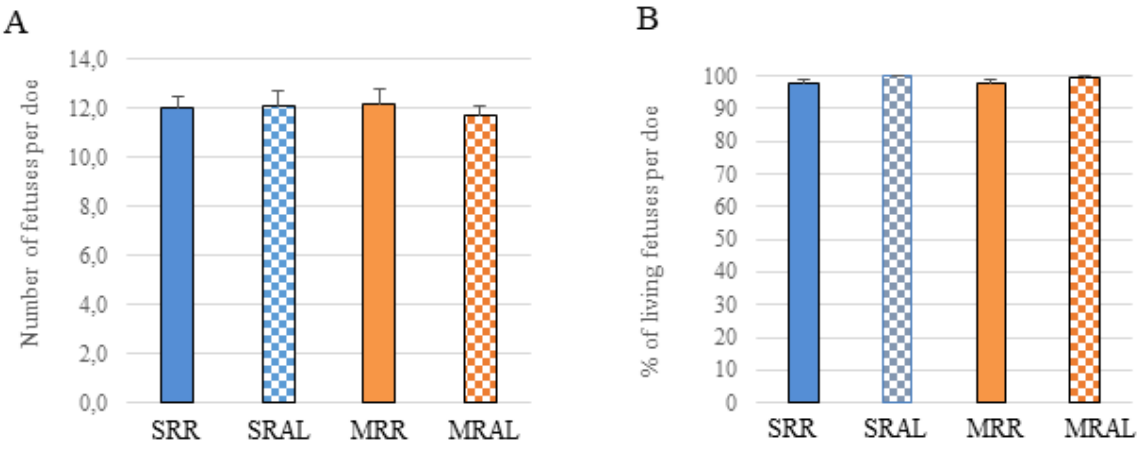


Figure 4 : (A) Average number of fetuses per doe in each group (n=10). (B) Average percentage of living fetuses per doe in each group.

232

233

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).
244

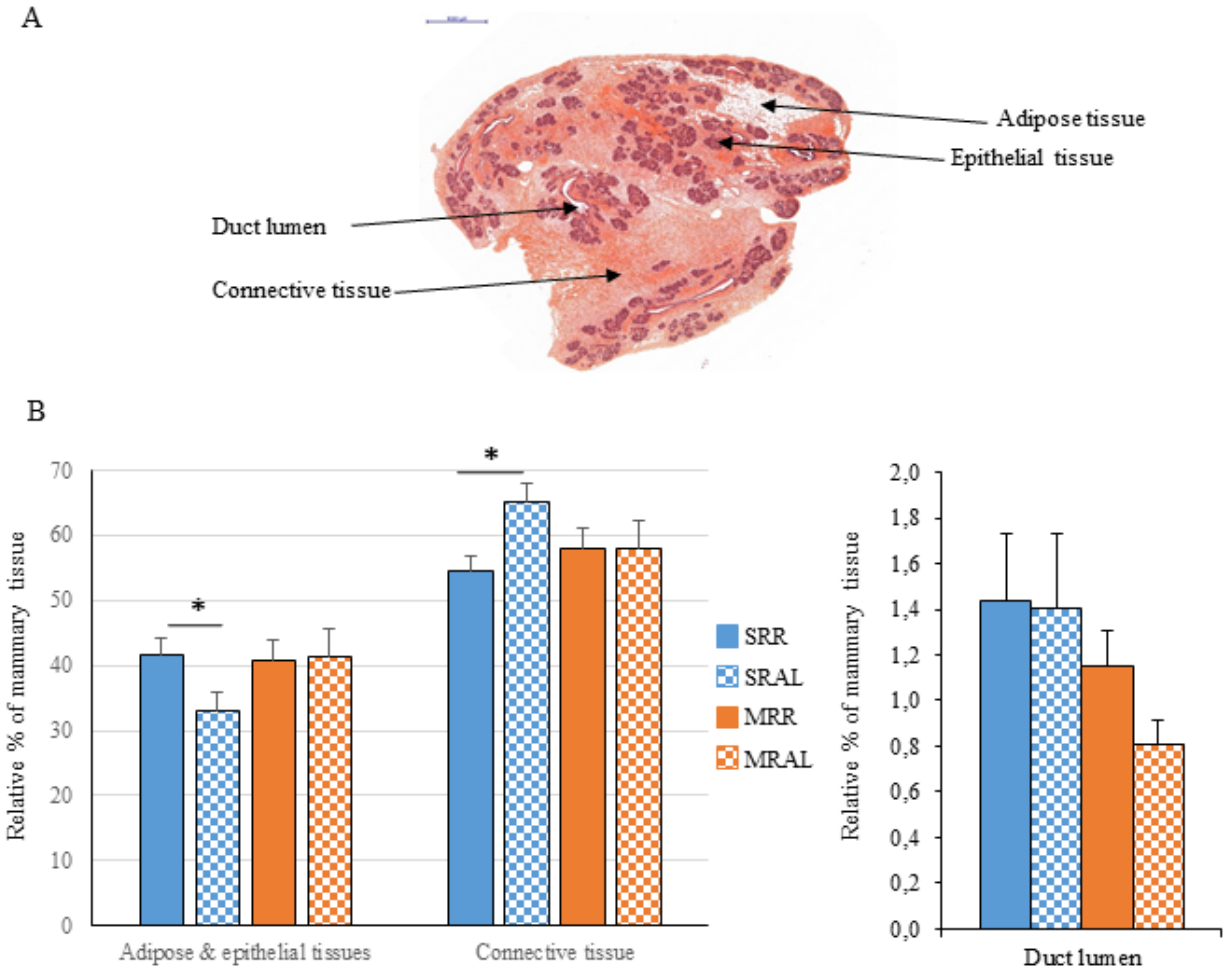
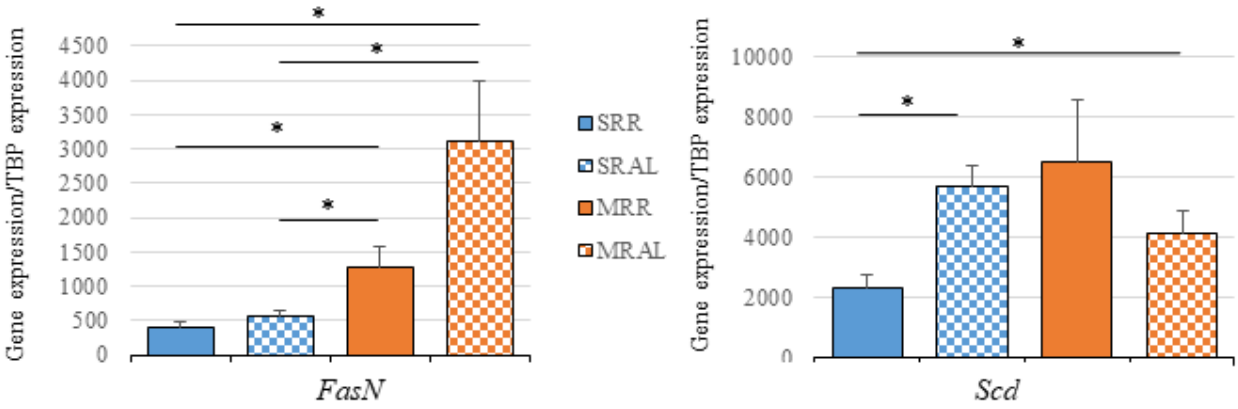


Figure 5: Histological analyses of mammary gland on Day 14 of pregnancy. (A) Representation of the different types of tissues present on a representative histological section of mammary gland, stained with hematoxylin and eosin. Scale bar represents 1mm (B) Relative quantification of mammary gland tissues: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

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

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



Figue 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 ± SEM. Significant differences (at least $P < 0.05$) between the groups are indicated by asterisks (*).

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

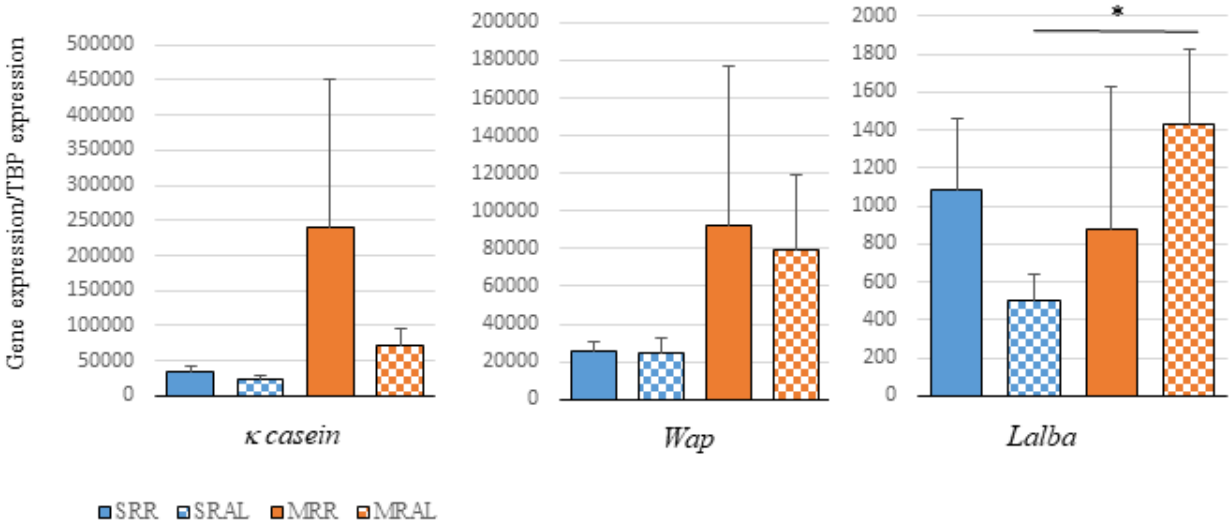


Figure 7: *K casein, Wap* and *Lalba* expression in mammary epithelial tissue on day 14 of pregnancy of the four groups of rabbits (n=10 in each group), according to the feeding strategies (groups SRR to MRAL). Transcripts levels were assessed for *κ casein, Wap* and *Lalba,* and normalized with *Tbp*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

DISCUSSION

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

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

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

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

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

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

No drastic changes in mammary gland histology were observed according to the different feeding strategies, which is reassuring since these strategies are based on restrictions practiced 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,

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

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

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

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

In rabbit breeding, feed restriction strategy is widespread used to improve productivity, decreased mortality and economic count. For fattening rabbits, feed restriction during rearing period allowed uniformity in body weight and decreased neonatal mortality (Rommers *et al.* 2001). While a moderate feeding restriction may improve some sperm morphologic characteristics, as well as fertility in male rabbits (Pascual *et al.* 2016), it seems that restriction strategies have shown less beneficial effects and even more for rabbit does (Birolo *et al.* 2020). Restricting feeding during different stages of pregnancy, even if it does not strongly affect growth of young rabbits, may delay placental growth, decrease the offspring survival and birth weight (Rommers *et al.* 2004; Matsuoka *et al.* 2012; Manal, Tony, and Ezzo 2010). In early life, rearing does with the same feeding strategies as fattening rabbits, leads to energy deficit, body mobilization and may reduce reproductive performance (Fortun-Lamothe 2006). Consequently, feeding strategies and lifespan are closely linked in rabbit does.

Our findings showed that feed restriction strategies applied during post-weaning and/or pubertal period can impact mammary gland structure and may delay mammary epithelial tissue development and functionality. These results also suggest the urgent need of further investigations on milk composition and subsequent lactation capacity of restricted does through several reproductive cycles to provide recommendations. Indeed, while a moderate feeding restriction does not necessarily have consequences, a severe one could adversely affect health and breeding performances.

411 **REFERENCES**

412 Angelucci, C., A. D'Alessio, F. Iacopino, G. Proietti, A. Di Leone, R. Masetti, and G. Sica.
413 2018. 'Pivotal role of human stearoyl-CoA desaturases (SCD1 and 5) in breast cancer
414 progression: oleic acid-based effect of SCD1 on cell migration and a novel pro-cell
415 survival role for SCD5', *Oncotarget*, 9: 24364-80.

416 Bautista, C. J., G. L. Rodriguez-Gonzalez, N. Torres, R. Hernandez-Pando, V. Ramirez, M.
417 Rodriguez-Cruz, P. W. Nathanielsz, and E. Zambrano. 2013. 'Protein restriction in the
418 rat negatively impacts long-chain polyunsaturated fatty acid composition and
419 mammary gland development at the end of gestation', *Arch Med Res*, 44: 429-36.

420 Birolo, M., A. Trocino, A. Zuffellato, and G. Xiccato. 2020. 'Effects of time-based feed
421 restriction on morbidity, mortality, performance and meat quality of growing rabbits
422 housed in collective systems', *Animal*, 14: 626-35.

423 Farmer, C. 2018. 'Nutritional impact on mammary development in pigs: a review', *J Anim Sci*,
424 96: 3748-56.

425 Farmer, C., M. F. Palin, and Y. Martel-Kennes. 2012. 'Impact of diet deprivation and
426 subsequent over-allowance during prepuberty. Part 1. Effects on growth performance,
427 metabolite status, and mammary gland development in gilts', *J Anim Sci*, 90: 863-71.

428 Farmer, C., D. Petitclerc, M. T. Sorensen, M. Vignola, and J. Y. Dourmad. 2004. 'Impacts of
429 dietary protein level and feed restriction during prepuberty on mammogenesis in gilts',
430 *J Anim Sci*, 82: 2343-51.

431 Fernandez-Twinn, D. S., S. Ekizoglou, M. S. Martin-Gronert, J. Tarry-Adkins, A. P.
432 Wayman, M. J. Warner, J. A. Luan, B. A. Gusterson, and S. E. Ozanne. 2010. 'Poor
433 early growth and excessive adult calorie intake independently and additively affect
434 mitogenic signaling and increase mammary tumor susceptibility', *Carcinogenesis*, 31:
435 1873-81.

436 Fleige, S., and M. W. Pfaffl. 2006. 'RNA integrity and the effect on the real-time qRT-PCR
437 performance', *Mol Aspects Med*, 27: 126-39.
438 Fortun-Lamothe, L. 2006. 'Energy balance and reproductive performance in rabbit does',
439 *Anim Reprod Sci*, 93: 1-15.
440 Gidenne, T., S. Combes, A. Feugier, N. Jehl, P. Arveux, P. Boisot, C. Briens, E. Corrent, H.
441 Fortune, S. Montessuy, and S. Verdelhan. 2009. 'Feed restriction strategy in the
442 growing rabbit. 2. Impact on digestive health, growth and carcass characteristics',
443 *Animal*, 3: 509-15.
444 Gidenne, T., S. Combes, and L. Fortun-Lamothe. 2012. 'Feed intake limitation strategies for
445 the growing rabbit: effect on feeding behaviour, welfare, performance, digestive
446 physiology and health: a review', *Animal*, 6: 1407-19.
447 Haschke, F., C. Binder, M. Huber-Dangl, and N. Haiden. 2019. 'Early-Life Nutrition, Growth
448 Trajectories, and Long-Term Outcome', *Nestle Nutr Inst Workshop Ser*, 90: 107-20.
449 Hue-Beauvais, C., P. Chavatte-Palmer, E. Aujean, M. Dahirel, P. Laigre, C. Pechoux, S.
450 Bouet, E. Devinoy, and M. Charlier. 2011. 'An obesogenic diet started before puberty
451 leads to abnormal mammary gland development during pregnancy in the rabbit', *Dev*
452 *Dyn*, 240: 347-56.
453 Hue-Beauvais, C., J. Laubier, N. Brun, I. Houtia, F. Jaffrezic, C. Bevilacqua, F. Le Provost,
454 and M. Charlier. 2019. 'Puberty is a critical window for the impact of diet on
455 mammary gland development in the rabbit', *Dev Dyn*.
456 Hulot, F., J. C. Mariana, and F. Lebas. 1982. '[Establishment of puberty in the doe rabbit
457 (folliculogenesis and ovulation. Effect of rationing the diet]', *Reprod Nutr Dev*, 22:
458 439-53.
459 Igal, R. A. 2011. 'Roles of StearoylCoA Desaturase-1 in the Regulation of Cancer Cell
460 Growth, Survival and Tumorigenesis', *Cancers (Basel)*, 3: 2462-77.

461 Jow, G. M., T. T. Yang, and C. L. Chen. 2006. 'Leptin and cholesterol levels are low in major
462 depressive disorder, but high in schizophrenia', *J Affect Disord*, 90: 21-7.
463 Livak, K. J., and T. D. Schmittgen. 2001. 'Analysis of relative gene expression data using
464 real-time quantitative PCR and the 2(-Delta Delta C(T)) Method', *Methods*, 25: 402-8.
465 Lu, M. H., and R. R. Anderson. 1973. 'Growth of the mammary gland during pregnancy and
466 lactation in the rabbit', *Biol Reprod*, 9: 538-43.
467 Macias, H., and L. Hinck. 2012. 'Mammary gland development', *Wiley Interdiscip Rev Dev*
468 *Biol*, 1: 533-57.
469 Manal, A. F., M. A. Tony, and O. H. Ezzo. 2010. 'Feed restriction of pregnant nulliparous
470 rabbit does: consequences on reproductive performance and maternal behaviour', *Anim*
471 *Reprod Sci*, 120: 179-86.
472 Martinez-Paredes, E., D. Savietto, L. Rodenas, C. Cervera, E. Blas, G. Brecchia, C. Boiti, and
473 J. J. Pascual. 2019. 'Consequences of rearing feeding programme on the performance
474 of rabbit females from 1st to 2nd parturition', *Animal*, 13: 2173-82.
475 Matsuoka, T., Y. Mizoguchi, R. Haneda, E. Otsuka, H. Mizuguchi, K. Fukuda, T. Ishikawa,
476 K. Tsurumoto, M. Noguchi, S. Tamai, and Y. Asano. 2012. 'Effects of restricted
477 feeding on fetal and placental development in pregnant rabbits', *J Toxicol Sci*, 37: 207-
478 14.
479 Neave, H. W., J. H. C. Costa, J. B. Benetton, D. M. Weary, and M. A. G. von Keyserlingk.
480 2019. 'Individual characteristics in early life relate to variability in weaning age,
481 feeding behavior, and weight gain of dairy calves automatically weaned based on solid
482 feed intake', *J Dairy Sci*, 102: 10250-65.
483 Olson, L. K., Y. Tan, Y. Zhao, M. D. Aupperlee, and S. Z. Haslam. 2010. 'Pubertal exposure
484 to high fat diet causes mouse strain-dependent alterations in mammary gland
485 development and estrogen responsiveness', *Int J Obes (Lond)*, 34: 1415-26.

Panzuti, C., C. Duvaux-Ponter, R. M. Bruckmaier, and F. Dessauge. 2019. 'Effect of feeding level during the prepubertal phase on mammary gland development in female goat kids', *J Dairy Res*, 86: 267-71.

Park, C. S., M. G. Baik, W. L. Keller, and W. D. Slanger. 1994. 'Dietary energy restriction-mediated growth and mammary development in rats', *J Anim Sci*, 72: 2319-24.

Pascual, J. J., F. Marco-Jiménez, E. Martínez-Paredes, L. Ródenas, C. Fabre, M. A. Juvero, and J. L. Cano. 2016. 'Feeding programs promoting daily feed intake stability in rabbit males reduce sperm abnormalities and improve fertility', *Theriogenology*, 86: 730-7.

Rizzoto, G., D. Sekhar, J. C. Thundathil, P. K. Chelikani, and J. P. Kastelic. 2019. 'Calorie Restriction Modulates Reproductive Development and Energy Balance in Pre-Pubertal Male Rats', *Nutrients*, 11.

Robinson GW, McKnight RA, Smith GH, Hennighausen L. 1995. 'Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation.', *Development.*, 121: 2079-90.

Rommers, J. M., B. Kemp, R. Meijerhof, and J. P. Noordhuizen. 2001. 'The effect of litter size before weaning on subsequent body development, feed intake, and reproductive performance of young rabbit does', *J Anim Sci*, 79: 1973-82.

Rommers, J. M., R. Meijerhof, J. P. Noordhuizen, and B. Kemp. 2004. 'The effect of level of feeding in early gestation on reproductive success in young rabbit does', *Anim Reprod Sci*, 81: 151-8.

Shankar, A., S. Syamala, J. Xiao, and P. Muntner. 2012. 'Relationship between Plasma Leptin Level and Chronic Kidney Disease', *Int J Nephrol*, 2012: 269532.

Shi, H., J. Zhu, J. Luo, W. Cao, H. Shi, D. Yao, J. Li, Y. Sun, H. Xu, K. Yu, and J. J. Loor. 2015. 'Genes regulating lipid and protein metabolism are highly expressed in mammary gland of lactating dairy goats', *Funct Integr Genomics*, 15: 309-21.

511 Stumpf, M. T., V. Fischer, C. M. McManus, G. J. Kolling, M. B. Zanela, C. S. Santos, A. S.
512 Abreu, and P. Montagner. 2013. 'Severe feed restriction increases permeability of
513 mammary gland cell tight junctions and reduces ethanol stability of milk', *Animal*, 7:
514 1137-42.
515 Suburu, J., L. Shi, J. Wu, S. Wang, M. Samuel, M. J. Thomas, N. D. Kock, G. Yang, S.
516 Kridel, and Y. Q. Chen. 2014. 'Fatty acid synthase is required for mammary gland
517 development and milk production during lactation', *Am J Physiol Endocrinol Metab*,
518 306: E1132-43.
519 Takeuchi, H., T. Nakamoto, Y. Mori, M. Kawakami, H. Mabuchi, Y. Ohishi, N. Ichikawa, A.
520 Koike, and K. Masuda. 2001. 'Comparative effects of dietary fat types on hepatic
521 enzyme activities related to the synthesis and oxidation of fatty acid and to lipogenesis
522 in rats', *Biosci Biotechnol Biochem*, 65: 1748-54.
523 Tian, H., J. Luo, Z. Zhang, J. Wu, T. Zhang, S. Busato, L. Huang, N. Song, and M. Bionaz.
524 2018. 'CRISPR/Cas9-mediated Stearoyl-CoA Desaturase 1 (SCD1) Deficiency Affects
525 Fatty Acid Metabolism in Goat Mammary Epithelial Cells', *J Agric Food Chem*, 66:
526 10041-52.
527 Tsiplakou, E., E. Flemetakis, E. D. Kouri, K. Sotirakoglou, and G. Zervas. 2015. 'The effect
528 of long term under- and over-feeding on the expression of genes related to lipid
529 metabolism in mammary tissue of sheep', *J Dairy Res*, 82: 107-12.
530 Tumova, E., Z. Volek, D. Chodova, H. Hartlova, P. Makovicky, J. Svobodova, T. A. Ebeid,
531 and L. Uhlirova. 2016. 'The effect of 1-week feed restriction on performance,
532 digestibility of nutrients and digestive system development in the growing rabbit',
533 *Animal*, 10: 1-9.
534 Villeneuve, L., D. Cinq-Mars, and P. Lacasse. 2010a. 'Effects of restricted feeding of
535 prepubertal ewe lambs on growth performance and mammary gland development',
536 *Animal*, 4: 944-50.

Villeneuve, L., D. Cinq-Mars, and P. Lacasse.. 2010b. 'Effects of restricted feeding of prepubertal ewe lambs on reproduction and lactation performances over two breeding seasons', *Animal*, 4: 1997-2003.

Wang, R., M. Kuang, H. Nie, W. Bai, L. Sun, F. Wang, D. Mao, and Z. Wang. 2016. 'Impact of Food Restriction on the Expression of the Adiponectin System and Genes in the Hypothalamic-Pituitary-Ovarian Axis of Pre-Pubertal Ewes', *Reprod Domest Anim*, 51: 657-64.

Zened, Asma, Bertrand Méda, Paul Ponchant, Aurélie Wilfart, J. Arroyo, Thierry Gidenne, Sylvie Combes, and L. Fortun-Lamothe. 2013. *Conséquences d'une restriction alimentaire chez le lapereau sevré sur les impacts environnementaux de la production de viande de lapin*.