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

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Running head: Feeding strategies influence mammary development

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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.

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).

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MATERIALS AND METHODS

Animals, experimental design and sampling

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

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

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;

Sampling and metabolic assays

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

Histological analysis

For histology, samples were fixed for 24 hours in RCI2 buffer (Alphelys, France) before embedding in paraffin. Five-micrometer sections, separated at least by 100 μ m 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 $\pm$ 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- $\Delta\Delta C_t$ 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'
<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	GGTCTCTGCTGGCTTGTTTC
<i>TATA Binding protein (Tbp)</i>	Forward	TGACCCCCATGACCCCTATT
	Reverse	CAGCAAACCGCTTGGGATTA

Statistical analyses

Experimental data are presented as means $\pm$ 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

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

decrease in cholesterol levels (Fig 3). This difference was not observed among does which were 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 (*).

The feeding strategies had no effect on the reproductive parameters: the fertility rate (data not 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.

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

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

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 $n = 10$.

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).

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 (*).

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 $\pm$ 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

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

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

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

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

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

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