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FEED INCORPORATION OF DEHYDRATED SAINFOIN: EFFECTS ON HEALTH AND PERFORMANCES OF DOES AND GROWING RABBITS

Cecile Gayrard*§, Antoine Bretaudeau†, Pascale Gombault‡, Hervé Hoste§, Thierry Gidenne*

*GenPhySE, Université de Toulouse, INRAE, ENVT, F-31326, Castanet Tolosan, France.
†Arrivé-Bellanné, Nueil-les-Aubiers, France.
‡Multifolia, Viapres-le-petit, France.
§Interaction Hôte-Agents Pathogènes, INRAE, ENVT, 31300 Toulouse, France.

Abstract: The performance and health of does and growing rabbits were compared over three consecutive reproductive cycles for three groups of 20 nulliparous does and their litters (DS0, DS13, DS26) fed isonutritive feeds containing 0, 13 or 26% dehydrated sainfoin (DS, Perly cultivar). Feed intake, live weight and fertility of does were not affected by DS feed incorporation. The number of live kits at birth increased linearly with increasing DS incorporation (+1.5 from DS0 to DS26, P=0.042) and the stillborn rate tended to linearly decrease in groups fed DS (16.6 *vs*. 10.4%, P=0.086). Increasing the level of DS in feeds had no impact on the growth of the kits before weaning, but led to a linear reduction in the post-weaning growth rate (P<0.01, –2 for 26% DS), whereas the feed conversion ratio increased linearly with DS incorporation (P<0.01, 2.91 *vs*. 2.98, resp. for DS0 and DS26). No effect of DS feed incorporation was detectable on doe and kit mortality rates. Excretion of coccidia by both does and growing rabbits was not affected by DS incorporation. For 70 d old rabbits, the levels of immunoglobulins A and G and of white blood cells were not significantly different between groups and high levels of IgG (average: 8.1 mg/mL) were recorded, suggesting a coccidia infestation. Overall doe mortality remained under 5% and was not affected by the reproductive cycle (P=0.24). The stillborn rate decreased from 18 to 6%, (P<0.01) from cycle 1 to 2, and the number of live rabbits at birth increased from 8.0 to 10.7 (P<0.01). Kit mortality remained low before weaning (under 2.5%), and very low after weaning (<1%). Excretion of coccidia by does decreased from cycle 1 to cycle 3, whereas excretions by growing rabbits remained stable.

Key Words: dehydrated sainfoin, rabbit feeding, growth, reproductive performance, coccidiosis, immunity.

INTRODUCTION

To cover the nutritional needs of rabbits, the feed must contain not only energy and protein, but also fibres, particularly lignins, which are required for their digestive health (Gidenne *et al.,* 2020). Sainfoin (*Onobrychis viciifolia*) is a legume traditionally planted in pasture, and used as hay or silage for livestock (Aufrère *et al.,* 2013). Sainfoin seems a good candidate for rabbit feed, as it contains relatively high levels of digestible proteins and energy (Gayrard *et al.,* 2021), as well as a high concentration of fibre and especially of lignins (Legendre *et al.,* 2018). Today, sainfoin is available as dehydrated pellets (Multifolia company, Perly cultivar) and its nutritive value for growing rabbits was recently studied according to the cut (Gayrard *et al.,* 2021). However, no information is available on the optimal incorporation level of dehydrated sainfoin (DS) pellets in balanced feed and its effect on the performance and health of growing rabbits and does.

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Moreover, sainfoin could be an alternative to the alfalfa classically used as a fibre source. Sainfoin also contains phenols, including condensed tannins (CTs), which could contribute to the control of digestive parasitism and thus help reduce the use of anthelmintic and anticoccidials (Hoste *et al.,* 2015). Two studies on the role of DS (Perly cultivar) in controlling parasitic infestations in growing rabbits have shown encouraging results (Legendre *et al.,* 2017, 2018). Tannins also have antimicrobial properties and could influence immune responses (Mueller-Harvey *et al.,* 2019).

Thus, our study aimed to assess the effect of the DS incorporation rate (0, 13, 26%) in balanced diets on performance and health of does and growing rabbits over three consecutive reproductive cycles.

MATERIALS AND METHODS

The experiments were conducted at the INRAE experimental farm (Pectoul, INRAE, Castanet-Tolosan, France) in accordance with French legislation on animal experimentation and ethics (authorisation number: SSA_2019_008), and the researchers were authorised by the French Ministry of Agriculture to conduct experiments on living animals.

Dehydrated sainfoin and experimental feed

DS was obtained from a first cut (Perly cultivar) harvested in May 2019, and provided as pellets by Multifolia company (Viapres-le-Petit, France). Three reproductive "R" diets and three fattening "F" diets (Table 1) were formulated to meet the requirements of reproductive does and growing rabbits, respectively (Gidenne *et al.,* 2015). Within a category (R or F), diets differed mainly in their DS incorporation rate (0, 13 or 26%, in R0 and F0, R13 and F13 or R26 and F26, respectively) but were isonutritive (crude protein, fibres and digestible energy). The six feeds were manufactured in one batch by Arrivé-Bellanné Company (Nueil-les-Aubiers, France). The diets contained no drugs or coccidiostat supplementation.

Animals and experimental design

The trial was carried out in three consecutive reproductive cycles (Figure 1), starting with 70 nulliparous does (genotype INRAE 0171) of similar weight at the start of the first cycle (insemination 1 at 20 weeks old). All the

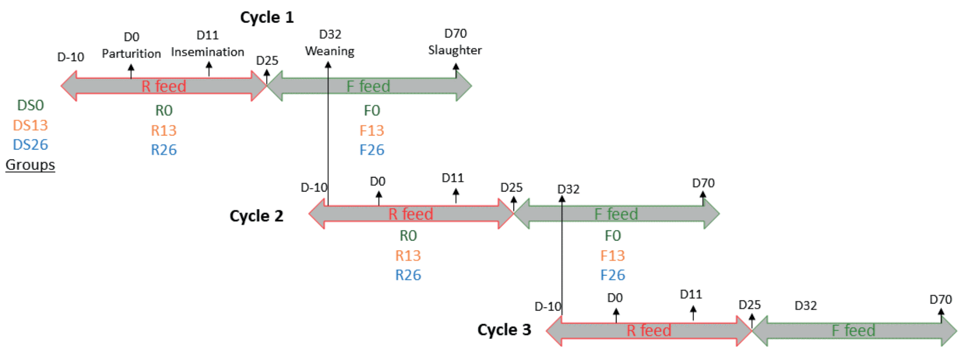

Figure 1: Experimental design. R feed was given to does and their litters from D-10 to D25. Does and litters were fed F feed from D25 to D32. From D32 to D70, growing rabbits were kept in fattening pens and fed F feed. At D32, does began cycle 2 and were again fed R feed. Measurements made of does: live weight and feed intake measured at D-10, D4, D14, D24 and D32; faecal samples collected at D-5, D2, D9, D16, D23 and D30. Measurement of litters: live weight measured at D4, D14, D24 and D32. Measurements of growing rabbits: live weight measured at D32, D49 and D70; intake refusal checked daily; faecal collections at D38, D45, D52, D59, D66. Cycle 1 started on 09/27/2019 (D-10); cycle 2 started on 11/08/2019; cycle 3 started on 12/20/2019. DS incorporation rate in feed (0, 13 or 26%, in R0 and F0, R13 and F13 or R26 and F26, respectively) .

Table 1: Ingredients and chemical composition of the diets and of dehydrated sainfoin.

	Diets of reproducing does			Diets of growing rabbits			Dehydrated sainfoin
Ingredients (g/kg)	R0	R13	R26	F0	F13	F26	1st cut 2019
Dehydrated sainfoin	0	130	259	0	156	260	
Wheat bran	217	249	250	110	223	269	
Soft wheat	110	114	90	24	50	32	
Beet pulp	106	94	88	242	196	158	
Rapeseed meal	80	76	80	70	74	90	
Sunflower meal	190	170	76	162	114	36	
Alfalfa meal	60	0	0	100	0	0	
Peas	56	44	58	30	0	0	
Maize	30	30	30	30	0	0	
Corn sprouts	30	20	30	20	0	0	
Treacle	22	10	10	15	10	10	
Grapeseed pulp	0	0	0	46	20	0	
Lapilest [a]	58	24	0	114	136	132	
Mineral and amino acids [b]	41	39	29	37	21	13	
Chemical composition (g/kg as fed)							
Dry matter	887	890	891	886	891	893	894
Organic matter	735	742	723	738	740	747	745
Crude ash	152	148	168	148	151	146	149
Crude protein	172	180	171	142	154	155	158
Neutral detergent fibre	400	370	374	466	460	452	421
Acid detergent fibre	158	167	167	227	223	227	294
Lignins	45	57	59	85	87	95	124
Digestible energy, kcal [c]	2618	2620	2621	2332	2335	2335	–
Total phenols [d]	10.2	9.8	11.8	9.0	11.8	12.0	29.4
Total tannins [d]	<5	<5	<5	<5	<5	<5	17.4

[a]Lapilest® is a commercial product containing a mix of fibrous materials such as grape pulp, beet pulp, sunflower hulls, grape seeds, dehydrated apple pomace, grape seed meal.
[b]Mineral and amino acids provided per diet: Lysine (0.71%), methionine (0.23%), threonine (0.64%), tryptophan (0.22%), isoleucine (0.74%), valine (0.85%), arginine (0.68%), phosphorus (0.28%), calcium (2.6%), sodium (0.03%) and potassium (2.42%).
[c]Digestible energy in each feed was calculated from the concentration of digestible energy in each ingredient.
[d]Analysed by Inovalys, Nantes, France.

animals (does and their litters) were housed in wire net cages (length: 68 cm, width: 62 cm, height: 48 cm). Ten extra does were kept for cycle 2 or 3 to replace does that died or were infertile. Ten days (D-10) before parturition (D0, Figure 1), 60 does were allotted to three equal groups based on diets: DS0 (control group), DS13 and DS26, with *ad libitum* access respectively to the feeds R0, R13 and R26. Litters were equalised at 8 kits at D4 and had access to "R" feed until they were 25 d old. At 25 d old (D25), litters and does of groups DS0, DS13 and DS26 fed R0, R13 and R26 respectively were switched to a fattening "F" feed F0, F13 and F26, with *ad libitum* access until weaning (D32). At weaning, the three groups of litters were moved to a fattening unit. They were housed collectively (6 rabbits per cage: length: 68 cm, width: 62 cm, height: 48 cm) and were fed the same F feeds (according to their group) until 70 d old, but under a restriction programme: 90 g per rabbit during the week after weaning, then increased feed distribution at a rate of +20 g/wk. At weaning, the does remained in their cage and were again fed R feed (with respect to their group) to start their 2nd cycle (Figure 1). The third cycle was performed in the same conditions. At the end of the third cycle, 20 growing rabbits per group (DS0, DS13 and DS26) were slaughtered at 70 d old.

Mortality and performance measurements

Mortality was checked daily. When a doe died, her entire litter was removed from the experiment. In each cycle, the live weight of each doe was recorded at D-10, D4, D14, D24 and D32, while the litters (8 kits/doe=160 kits/group) were weighed at D4, D14 and D24. Feed intake of doe and litters was checked on the same dates as doe live weight. The live weight of each growing rabbit (120 rabbits/group) was recorded at weaning (D32), and at 49 and 70 d old. The feed intake of the growing rabbit was checked weekly in each cage. Reproductive performances including insemination rate, the number of kits alive at birth, and the number of stillborn were measured at each cycle.

Faecal sampling for coccidia concentration measurement

Faeces were collected once a week from a 1 cm mesh attached under the cages with 6 collections (at D-5, D2, D9, D16, D23 and D30) from cages containing does, and 5 collections (at D38, D45, D52, D59, D66) from cages containing growing rabbits. Faeces were collected from does (1 doe per cage=1 sample of faeces per doe) and for 10 does per group. Faeces from growing rabbits were collected from 10 cages, each cage containing 6 growing rabbits (1 sample corresponded to faeces collected from 6 growing rabbits). Faeces were stored at 4°C for one day prior to oocyst counting at the Toulouse National Veterinary School (ENVT, France). The concentration of faecal oocysts (CFO) was assessed using the modified McMaster technique (Gibbons *et al.,* 2005) and is expressed as the number of oocysts per gram (OPG) of fresh faeces. A mean CFO value was calculated for each cage for the entire faecal collection period (5 collections).

Sampling of blood and digesta from growing rabbits

Twenty healthy growing rabbits of similar weight per group (DS0, DS13 and DS26) in cycle 3 were sacrificed at 70 d old by electrical stunning followed by exsanguination. Blood was collected from the aorta in two heparinised tubes (Vacuette, 9ml NH Sodium Heparin) and immediately stored on ice. One tube was used to measure total white blood cell counts and the diversity of white blood cell types with a haemocytometer (MS9-5 VET, Malet Schloesing, Osny, France). The second tube was centrifuged, and plasmas were stored at –20°C until analysis of immunoglobulins. Faeces were collected from the rectum of euthanised rabbits and stored at –20°C until analysis. Caeca were isolated, weighed and sampled and then stored at –20°C for analysis.

Analysis of immunoglobulins: extraction and ELISA measurements

ELISA was used to quantify IgG in plasma and the concentration of IgA in caecum digesta and in faeces. In a preliminary step, caecal content was diluted to 50 mg/mL in TBS buffer while a small amount of faecal matter (200-600 mg) was diluted at 25 g/L in PBS buffer. After thorough shaking, the samples were centrifuged at 3000 g for 10 min at 4°C. The supernatants were collected and stored at –20°C until analysis. Total IgG or IgA contents were determined in triplicate by sandwich ELISA in 96-well plates coated with specific polyclonal goat anti-rabbit IgG or IgA antibody (Bethyl Laboratories, Montgomery, Texas, USA). Appropriate sample dilutions were performed: from 1:64 000 to 1:128 000 for IgG, 1:10 for caecal IgA and from 1:7 to 1:50 for faecal IgA determination. Plates were read at 450 nm using a GloMax® Discover plate reader (Promega, Madison, WI) to construct the standard curve based on a four-parameter model. IgG in plasma was quantified using a reference IgG serum (Bethyl Laboratories, Montgomery, Texas, USA). Regarding caecal and faecal IgA, respectively sixty caecal and faecal samples were pooled to build a reference sample to construct standard curves. Relative IgA concentrations were then interpolated from this curve. Only results with intra-assay coefficients of variation <10% were kept (Wong *et al.,* 2004).

Chemical analyses of diets

Chemical analyses of dehydrated sainfoin and of the different feeds were performed at INRAE. Dry matter (DM) content was determined after drying at 103°C for 24 h and ash content after heating at 550°C for 5 h. Crude proteins were analysed using the Dumas combustion method. The “Van Soest” detergent procedure was used to analyse fibre fractions (aNDFom, ADFom and lignins) (Van Soest *et al.,* 1991) in the feed and in the dehydrated sainfoin. Total phenols and tannins were analysed in the feed and in dehydrated sainfoin using the Folin-Ciocalteu method (Council of Europe, 2007; Makkar, 2000) (Inovalys, Nantes, France). In sainfoin, total tannins correspond almost entirely to condensed tannins (Wang *et al.,* 2014; Mueller-Harvey *et al.,* 2019).

Statistical analyses

Data were first screened to detect outliers. No outlier was found for animal growth and feed intake. All data were analysed using R (R Core Team, 2020). A Shapiro-Wilk test was used to check normality. A two-factor (group and cycle, and interaction) variance analysis model was used to estimate the effect of the group (DS0, DS13, DS26) and that of the cycle (cycle 1, 2 or 3) on performance (live weight and growth) and reproductive traits (number of live rabbits). For each cycle, a single factor variance analysis was used to estimate the effect of diet on health traits (proportion of blood cells and concentrations of immunoglobulins). A Tukey's multiple means comparison test was used to compare means between the different groups and cycles. A Chi-Square test was used on mortality and fertility rates. A generalised linear model with the two above mentioned factors was used to analyse stillborn rates and faecal oocyst concentrations. A linear regression model was used to check the effect of the DS incorporation rate (0, 13, 26%) on the number of live rabbits at birth, on the post-weaning daily weight gain, and on the feed conversion ratio. Finally, another linear regression model was used to check the evolution of oocyst excretion in growing rabbits from D38 to D66.

RESULTS

Diets and sainfoin chemical composition

We used a Perly sainfoin from a first cut harvested in 2019 (crude protein=158 g/kg, acid detergent fibre=294 g/kg, crude fibre=17.4 g/kg) for F and R diets. The R and F diets were formulated to meet the requirements of reproductive does and growing rabbits, respectively. Within each feed category (R or F) the diets were isonutritive and contained similar levels of crude proteins, fibres, and digestible energy. Incorporation of dehydrated sainfoin in the R diets was achieved mainly at the expense of sunflower meal, alfalfa meal and Lapilest®; whereas in the F diets, DS mainly replaced beet pulp, sunflower meal, alfalfa meal and grapeseed pulp (Table 1). The F diets had higher concentrations of ADFom (+27%) and lignins content (+39%) than the R diets. Diets for growing rabbits were 14% poorer in crude proteins and 11% poorer in digestible energy than R diets. With increasing rates of sainfoin incorporation, the levels of phenols logically increased from 10 to 12 g/kg and from 9 to 12 g/kg in the R and F feeds, respectively.

Reproductive performance

No interaction between cycles and diets was found for reproductive performance of does (Table 2). As expected, doe live weight increased (except at D32) by 7% from cycle 1 to cycle 3 (Table 2, $P<0.001$), since they reached their adult weight. Throughout the study, the live weight of the does was not affected by the diet. The feed intake of does (and of litters before 25 d of age) increased by 20% ($P<0.05$) from cycle 1 to cycle 3 (Table 2), while it was not affected by the diets and averaged 356 g/d. The fertility rate remained similar between cycles and diets and averaged 78%. The number of live kits at birth was lower in cycle 1 (-2 live rabbits at birth) than in cycle 2 and 3 ($P<0.001$), and was similar in all three groups (9.5 live kits per doe, $P=0.067$). However, the number of liveborn kits increased linearly with the DS incorporation ($P=0.042$), resulting in +1.5 live rabbits at birth in DS26 group. From cycle 1 to cycle 2, the stillborn rate decreased by 67%, then increased again by 13% in cycle 3. The stillborn rate was similar among the three diets ($P=0.18$) and averaged 13%. However, when we compared groups with sainfoin diets (DS13+DS26) with the control group (DS0) in a contrast analysis, the stillborn rate tended to be lower in groups (10.4%, $P=0.086$) compared to DS0 (17%).

Feed intake by litters and does (D25-D33) increased by 15% from cycle 1 to cycle 3, and litter growth was 9% lower in the first cycle than in the two others ($P<0.05$). Feed intake by the litter and the doe was not affected by the DS dietary incorporation rate (mean: 799 g/d). Similarly, between 4 and 33 d of age, litter growth was not affected by the diet and averaged 28.8 g/day.

Performance of growing rabbits after weaning.

No interaction between cycle and diet was found for the performance of the growing rabbits (Table 3). Growing rabbits in all three groups were subject to similar restrictions from weaning (D33) until D70, and the feed intake averaged

Table 2: Reproductive performances of does according to cycle and sainfoin dietary incorporation.

	Cycles				Groups				*P*-value		
Doe	1	2	3	RSD	DS0	DS13	DS26	RSD	Cycle	Groups	Cycle × Groups
Live weight (g)											
D-10[1]	5168[a]	5454[b]	5560[c]	35.0	5447	5372	5346	37.6	<0.001	0.45	0.85
D4	5031[a]	5298[b]	5408[c]	30.9	5296	5282	5159	33.4	<0.001	0.13	0.74
D32	5225	5383	5348	37.5	5366	5358	5239	37.6	0.18	0.28	0.60
Doe feed intake:											
D-10 to D25 (g/d)	319[a]	365[b]	384[c]	3.83	364	352	351	4.48	<0.001	0.31	0.88
Litter+doe feed intake:											
D25-D32 (g/d)	737[a]	796[b]	866[c]	6.7	816	790	792	8.1	<0.001	0.18	0.44
Fertility rate (%)	80.0	71.9	81.4	3.02	78.9	76.4	77.6	3.03	0.40	0.95	0.99
Live kits at birth	8.0[a]	10.7[b]	10.0[b]	0.3	9.0	9.0	10.5	0.3	<0.01	0.067	0.61
Stillborn rate (%)	17.9[b]	5.9[a]	12.7[ab]	1.8	16.6	11.8	9.0	1.8	<0.01	0.18	0.47
Litter daily weight gain:											
D4-D32 (g/d)	27.2[a]	29.3[b]	30.2[b]	0.2	29.4	28.2	28.9	0.2	<0.001	0.10	0.87

[1]D-10: 10 d before parturition (D0); D4: 4 d after parturition; D32: 32 d after parturition.
N=20 does/diet. RSD: Residual standard deviation.
[abc]Means within a row and treatment with different superscript letters differ ($P<0.05$).

127 g/d/rabbit. No feed was refused whatever the diet. The growth rate and feed conversion improved from cycle 1 to cycle 3, respectively +14 and –14%. Increasing the sainfoin incorporation in the diets linearly reduced the growth rate (linear effect: $P<0.01$), resulting in a 2.3% lower growth rate ($P<0.001$) in the DS26 group compared to the DS0 group (Table 3). Accordingly, the feed conversion ratio linearly increased with the DS incorporation (linear effect: $P<0.01$), resulting in 2.3% higher feed conversion in group DS26.

Mortality of does and growing rabbits

Doe mortality was studied on a mean of 48 does per cycle instead of 60 does per cycle due to a 78% fertility rate and to gestating does dying before beginning the trial (2%). Doe mortality was low (5%) and not affected by the reproductive cycle (Table 4). However, doe mortality differed among groups, with higher mortality in group DS13 (15%) than in DS0 (0%) or DS2p6 (2%). Indeed, when doe losses were cumulated over the 3 cycles, six does out of the seven does that died belonged to group DS13. During cycle 1, four does died (at D0, D7 and D15) and three out of the four belonged to group DS13; veterinary diagnosis identified two pathogenic agents: *Escherichia coli* and *Staphylococcus aureus*. Consequently, from D18 to D24 (cycle 1), all the does were treated with 70 g/d of doxycycline, and from D25 to D30 (cycle 1) does received 0.5 ml/L of colistin in their drinking water. Thereafter, except for three does in group DS13 that died between 23 and 28 d in cycle 2, no more does died. The does that died in cycle 1 died abruptly with no sign of poor sanitary status (normal live weight, intake and body condition) and

Table 3: Performances of growing rabbits after weaning, according to the cycle and sainfoin dietary incorporation.

g/d	Cycles				Groups				*P*-value		
D32-D70	1	2	3	RSD	DS0	DS13	DS26	RSD	Cycle	Groups	Cycle X Groups
Feed intake	126.9	127.1	127.2	1.7	127.2	127.1	127.0	1.7	0.997	0.999	1
Daily weight gain	39.8[a]	44.7[b]	46.3[c]	0.1	44.2[b]	43.4[ab]	43.2[a]	0.2	<0.001	<0.001	0.76
Feed conversion ratio	3.22[c]	2.87[b]	2.76[a]	0.01	2.91[a]	2.97[ab]	2.98[b]	0.01	<0.001	<0.01	0.72

RSD: Residual standard deviation.
[abc]Means within a row and treatment with different superscript letters differ ($P<0.05$).
N=104 in DS0, 70 in DS13 and 100 in DS26.

Table 4: Mortality of does and growing rabbits according to the cycle and to sainfoin dietary incorporation.

	Cycles			Groups			*P*-value		
	1	2	3	DS0	DS13	DS26	Cycle	Groups	Cycle × Groups
Does	4/50	3/46	0/48	0/52[a]	6/41[b]	1/51[a]	0.24	<0.01	0.20
Growing rabbits									
Birth-weaning	11/392 (2.8%)	9/368 (2.4%)	3/384 (0.8%)	8/408 (2.0%)	8/320 (2.5%)	7/416 (1.7%)	0.11	0.73	<0.001
Weaning-70d old	1/294	1/276	3/288	1/312	2/240	2/306	0.42	0.72	0.28

[ab]Means within a row and treatment with different superscript letters differ ($P<0.05$).

with no possible relationship with the diet (or feed intake). Two does (out of three) that died in cycle 2 had lower live weights (–10%, –21%) than healthy does.

The mortality rate of the kits before weaning (D4 to D33) was generally low (mean 2.0%, Table 4), but a significant interaction was detected between the cycle and the diet (Figure 2). For instance, kit mortality in cycle 1 and cycle 3 averaged 2.6% and no significant difference was found among the diets. Reversely, during cycle 2, kit mortality was higher in DS13 group (6.7%) than with the DS0 (0.7%) and DS26 (0.8%) groups ($P=0.013$). Accordingly, during cycle 2, seven out of nine (77%) dead kits were in group DS13. Interestingly, most doe and kit mortality occurred among animals fed the DS13 group.

Post-weaning mortality (from D33 to D70) was very low (<1%) and was not affected by either the cycle or the group.

Oocyst excretion by does and growing rabbits

The faecal concentration of oocysts (CFO) showed a tenfold reduction between cycle 1 and cycle 2 ($P<0.001$, Table 5). In cycle 3, the mean concentration of faecal oocysts was almost null, but the CFO did not differ significantly from that in cycle 1 and 2 due to a high oocyst excretion variability (LCL=1; UCL=4 000). Conversely, the faecal concentration of oocysts in growing rabbits remained high throughout the study (cycle 1 to cycle 3, mean: 2 914 OPG). Throughout the study, the CFO in growing rabbits was higher than in does (respectively 2914 *vs.* 1 399 OPG, $P<0.05$. The faecal concentration of oocysts in does and growing rabbits was similar among the three groups ($P>0.40$, Table 5).

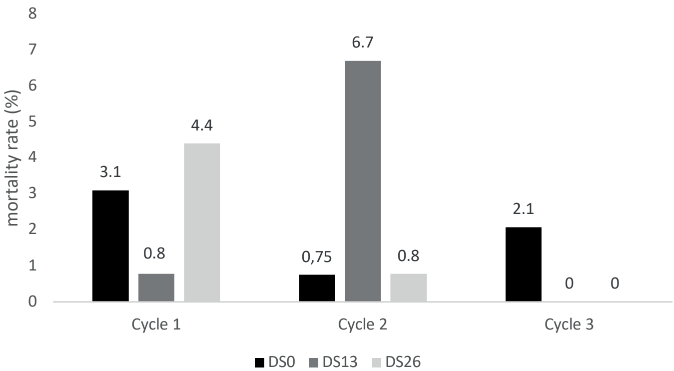

Figure 2: Kit mortality before weaning (D33) according to cycle and dehydrated sainfoin (DS) dietary incorporation. a,b: Within a cycle, means with different superscript letters differ ($P<0.05$). DS0: Group fed a R0 control diet without incorporation of dehydrated sainfoin pellets. DS13: Group fed a R13 diet containing 13% dehydrated sainfoin pellets. DS26: Group fed a R26 diet containing 26% dehydrated sainfoin pellets. ■ DS0; ■ DS13; ■ DS26.

Table 5: Faecal concentration of oocysts in does and growing rabbits according to the cycle and sainfoin dietary incorporation.

	Cycles				Groups				P-value		
OPG measures[1]	1	2	3	RSD	DS0	DS13	DS26	RSD	Cycle	Groups	Cycle X Groups
Does[2]	3844[a]	426[b]	67[b]	504	1539	2129	485	530	<0.001	0.30	0.64
Growing rabbits[3]	2766	3871	2105	382	3556	2371	2815	387	0.15	0.42	0.79

[1]Faecal concentration of oocysts expressed in oocysts per gram of faeces (OPG number).
[2]N=10 does per diet group.
[3]N=10 cages per diet group with 6 growing rabbits per group.
[ab]Means within a row and treatment with different superscript letters differ (P<0.05).

When the OPG levels of all growing rabbits were cumulated independently of cycles and diets, the oocysts faecal excretion showed a slight decrease (Figure 3) from D38 to D66, but without significance (P=0.14, R^2=0.45, RSD: 350).

Immune status of does and growing rabbits

White blood cell counts (WBC) were similar between diets (P=0.53, Table 6) and averaged 7.95 x 10^9/L. Among white blood cells, the proportions of lymphocytes, neutrophil and monocytes were 56, 32 and 8.5% respectively. The proportions of WBC types were similar in all three groups (P>0.05).

IgA levels measured in either caecal or faecal contents and plasmatic IgG levels were also similar among the three groups (P>0.05, Table 6). Total plasma IgG concentrations averaged 8.07 mg/mL.

DISCUSSION

Effect of incorporating sainfoin in rabbit feed.

Compared to another first cut of sainfoin (Gayrard *et al.,* 2021), our first cut sainfoin had a higher ADF concentration (208 *vs.* 294 g/kg as fed) and a much higher lignins level (64 *vs*. 124 g/kg as fed, Table 1), but lower concentration of crude proteins (200 *vs.* 158 g/kg as fed). However, compared to a sainfoin hay, our first cut sainfoin contained a higher (+14%) of crude proteins (Feedipedia). The phenol and tannin levels of our first cut were respectively 18% and

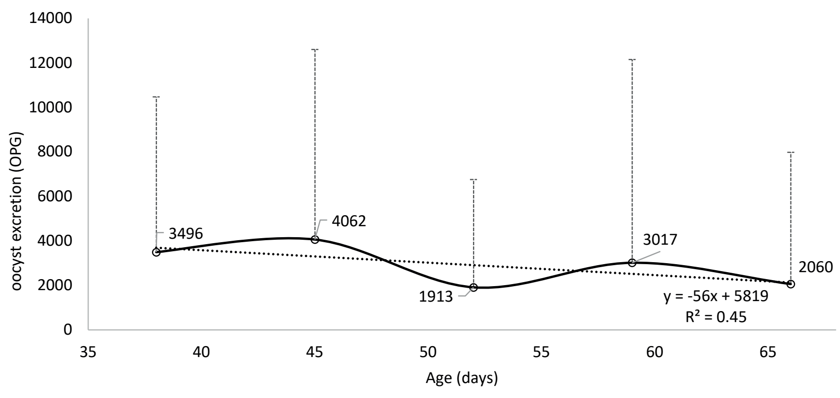

Figure 3: Number of coccidia oocysts excretion per gram of faeces according to age of the growing rabbit. Compiled data for the three reproductive cycles and for the three groups. Standard deviation of the mean represented for each mean excretion of oocysts/d. Linear regression model: P=0.14, and residual standard deviation: 350.

Table 6: White blood cells and immunoglobulins in growing rabbits (70 days old, cycle 3) according to sainfoin dietary incorporation.

	Groups				
	DS0	DS13	DS26	RSD	*P*-value
White blood cells, ($\times10^9$/L)	7.95	7.68	8.21	0.19	0.53
Lymphocytes (%)	57.16	55.54	54.38	0.88	0.41
Neutrophil (%)	30.45	31.81	34.24	0.91	0.21
Monocytes (%)	8.59	8.57	8.23	0.17	0.61
Eosinophil (%)	1.70	1.77	1.20	0.14	0.18
Basophil (%)	0.22	0.24	0.18	0.02	0.31
Immunoglobulins A and G					
Caecal IgA (AU)	1.34±0.78	1.81±1.83	1.06±0.54	0.15	0.46
Faecal IgA (AU)	0.16±0.17	0.18±0.34	0.16±0.13	0.03	0.61
Plasmatic IgG levels (mg/ml)	8.19±6.04	6.97±4.56	9.05±6.18	0.77	0.59

AU=arbitrary unit. Immunoglobulin levels expressed as mean±standard deviation. RSD: Residual standard deviation. N=20 growing rabbits per diet.

32% higher than for a first cut 2018 (Gayrard *et al.,* 2021), whereas the level of condensed tannins in a sainfoin hay was higher (30 g/kg, Feedipedia).

Sainfoin incorporation did not affect the fertility rate (mean 78%), which was in the normal range, and close to the value recorded on French rabbit farms (83%; Coutelet, 2015). With increasing incorporation of sainfoin, the number of live rabbits at birth increased linearly (P=0.042), while the stillborn rate decreased. This could be linked to the tannins supplied by the sainfoin. Indeed, in ewes fed *Lotus corniculatus* (rich in tannins) Min *et al.* (2001) observed a higher proportion of multiple ovulations and reduced embryonic loss, resulting in more lambs born per ewe.

Before weaning, neither feed intake (by the does and litters) nor litter growth rate were affected by the incorporation of sainfoin, whereas post-weaning growth and feed conversion were slightly impaired by sainfoin, particularly at the highest incorporation rate (26%). This effect could also be linked to the higher intake of total phenols and tannins that reduced protein digestion (Gayrard *et al.,* 2021).

Rabbits can be infected by several species of coccidia (hosted mainly in the digestive tract), and whether or not they develop the associated disease depends on the level of infection. In our trial, both does and growing rabbits were infected by coccidia, but with no visible signs of disease, such as diarrhoea or weight loss (Licois, 2004; Pakandl, 2009). In most coccidia species, the maximum number of oocysts per rabbit that can be excreted ranges from 1 to 5×10^8 (Pakandl, 2009), 10^5 times more than the coccidia excretion rate observed here (10^3). The antiparasitic properties of sainfoin (Hoste *et al.*, 2015) are related to its condensed tannin content (CTs) (Mueller-Harvey *et al.,* 2019; Wang *et al.,* 2014). CTs have anthelmintic properties, as already shown in sheep and goats (Hoste *et al.,* 2015), and are known to help limit coccidia infections in small ruminants (Saratsis *et al.,* 2012; Dykes *et al.,* 2019). Studies on growing rabbits fed a high percentage (40 and 34%) of pelleted dehydrated sainfoin reached similar conclusions for helminth (Legendre *et al.,* 2017) and coccidia control (Legendre *et al.,* 2018). In our study, no variation in coccidia infections was found as a result of a more moderate sainfoin incorporation. Considering that the level of infection in our study was already low, higher tannin intake may be needed for detectable antiparasitic effects.

Incorporating sainfoin in the diet did not modify either the blood formula or the proportion of white cells, which were within the physiological range (Moore *et al.,* 2015). Eosinophil is involved in the innate immune system, in particular in the phenomena of parasitism (Huang and Appleton, 2016). In the present study, despite the coccidia infection of growing rabbits, eosinophil levels did not increase above the physiological range, which may be explained by the relatively low level of coccidia infection. However, we could expect sainfoin intake to increase eosinophil levels, as sainfoin appears to have enhanced the innate immunity reaction against the parasite *Trichostrongylus colubriformis*. Indeed, in *Trichostrongylus colubriformis*-infested sheep fed sainfoin hay including 2% tannins, Ríos-de Álvarez *et al.* (2008) observed an increase in the proportion of eosinophil cells compared to those in sheep fed only grass. In our study, sainfoin diets had no effect on the proportions of eosinophil, which may be due to insufficient tannin intake.

Immunoglobulins levels were not modified by intake of sainfoin. However, total plasma IgG concentrations were twice as high as those measured by Knudsen *et al*. (2015). The similarly high level of IgG with normal IgA levels obtained in all groups of growing rabbits could be explained by the presence of coccidia. This result is in agreement with that of Pakandl *et al*. (2008), who observed an increase in IgG linked to IgG stimulation by parasite antigen with unchanged levels of IgA.

Effects of the reproductive cycle (age)

As expected, the live weight and feed intake of nulliparous does (cycle 1) were lower than those of multiparous does (cycle 2 and 3), but the fertility rate of nulliparous and multiparous does was similar. In commercial farms, nulliparous does often have lower fertility rates due to their incomplete body growth (Paci *et al.,* 2003), and Fortun-Lamothe (2005) observed a more negative energy balance during first lactation in primiparous does than in multiparous does. Thus, fertility rate would be better in primiparous does (not lactating) than in multiparous does that are inseminated while lactating at the same time. Fewer live rabbits were born to nulliparous does than to multiparous does (8 *vs*. 10.4 in cycles 2 and 3). Similarly, Castellini *et al*. (2006) and Theau-Clément *et al.* (2016) observed that nulliparous does had fewer live rabbits at birth (7 and 8.5 respectively) than multiparous does (8.3 and 11.0 respectively). In our study, the stillborn rate was high in cycle 1 (18%) and lower in cycle 2 (6%), in contrast to a study by Guillen *et al*. (2008), who observed a low stillborn rate at first parturition (11%) but an increase at the second parturition (20%). Theau-Clément *et al*. (2016) reported a low stillborn rate for both nulliparous and primiparous does (0.4 and 0.6 respectively), but the rate was nevertheless higher for multiparous does (1.0).

The growth rate and feed conversion performance of kits and growing rabbits improved with each cycle. For instance, Theau-Clément *et al*. (2016) reported a 15% lower growth rate for rabbits weaned by a primiparous doe compared to rabbits weaned by a multiparous doe. This is related with the higher milk production of a multiparous compared to a nulliparous doe.

In our experimental study, overall doe mortality was low (5%) from cycle 1 to cycle 3, close to the 3.8% mortality rate of does found by Rosell and Fuente (2016). Similarly, kit mortality before weaning was very low (2.0%). However, kit mortality in cycle 2 was unexpectedly higher and seemed related to the poorer health status of the does in this group. Indeed, it has frequently been reported that poor doe health status has a negative impact on the health of the litter (Rashwan and Marai, 2000). Surprisingly, in our study, kit mortality before weaning was higher than after weaning (<1%), in contrast to most previous studies in which post-weaning mortality is reported to be higher (Fortun-Lamothe and Gidenne, 2000; Theau-Clément *et al.,* 2016).

The decrease in doe oocyst excretion from the cycle 1 to the cycle 3 was most probably related to the development of immunity to oocyst infection. Norton *et al*. (1979) showed that a single inoculation with 100 oocysts of *E. flavescens* or *E. irresidua* was able to provide animals with slight protection against a second infection. Faecal concentrations of oocysts in growing rabbits were stable and moderate (<3000 OPG) throughout the trial, and remained below the threshold (4000-5000 OPG) for prophylactic treatment against coccidia (Coudert *et al.,* 2000). Indeed, young rabbits, especially from one to three months old, have low immunity to coccidia infection (Duszynski and Couch, 2013). Although coccidia infection was sharply reduced with doe age, this did not reduce the coccidia infestation of the litters. In fact, only a small amount of coccidia (80 to 200 oocysts) is sufficient to provide a high faecal infestation ($1\text{-}5\times10^8$) as shown by Pakandl (2009).

CONCLUSIONS

The incorporation of first-cut dehydrated sainfoin pellets in the feed of reproducing does did not modify their body status, feed intake or fertility rate. However, sainfoin in feed improved the number of live rabbits and tended to reduce the stillborn rate. After weaning, feed containing sainfoin slightly impaired growth and feed conversion, without affecting the health status. Incorporating sainfoin had no impact on the faecal concentration of coccidia or on immune status. Independently of sainfoin, the reproductive performances of the does (number of live rabbits at birth, stillborn rate) and growth of young rabbits improved from cycle 1 to cycle 3, while the excretion of coccidia decreased with age of the doe.

An incorporation of up to 26% sainfoin is thus possible in the feed of reproductive does. A moderate sainfoin incorporation (≤13%) in the feed would be preferred for young rabbit. These first results need to be confirmed using a larger number of rabbits raised in commercial conditions to assess the impact of incorporating sainfoin in feed on doe and litter health and performance.

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