

Is there any detrimental effect when a chestnut hydrolysable tannin extract is included in the diet of finishing lambs?

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Abstract – This work was conducted to ensure that the consumption of a small amount of a chestnut hydrolysable tannin (HT) extract, included in the diet (20.8 g·kg⁻¹ DM) of finishing lambs as a feed additive, does not cause toxic effects or does not negatively affect lamb performance. Thirteen Merino lambs were finished from 15 to 25 kg of live weight, which is the most typical slaughter weight for lambs in Spain. They were divided into two groups: one was used as the control (*Control*) and the other one received the treatment with tannins (*TAN*). The only difference between the groups was that the soya bean meal incorporated as the protein supplement in the *TAN* concentrate had been treated with the chestnut HT extract. No significant differences ($P > 0.10$) in voluntary intake, feed conversion, daily gain or length of fattening period were observed between the two groups. The histopathological examination showed no signs of toxicity due to the tannins. Likewise, the carcasses of the *TAN* group did not show residues of analysed HT metabolites (gallic acid, ellagic acid, resorcinol, pyrogallol and phloroglucinol). The raised activities of the enzymes GGT (gamma glutamyl-transferase) and AST (aspartate amino-transferase) in the *TAN* lambs suggest that experiments of longer duration need to be conducted, to further check for toxicity effects under these conditions.

feed additive / finishing lambs / hydrolysable tannins

Résumé – Y a-t-il des effets négatifs quand un extrait de tannins hydrolysables de châtaignier est inclus dans la ration de finition des agneaux ? La présente étude a été réalisée afin de s'assurer que la consommation de petites quantités d'un extrait de tannins hydrolysables (TH), inclus dans la ration d'agneaux en période de finition (20,8 g·kg⁻¹ DM) comme additif, n'engendre pas d'effets toxiques ou n'affecte pas négativement leurs performances. Treize agneaux de race Merinos ont été abattus entre 15 et 25 kg de poids vif, poids d'abattage typiques pour des agneaux en Espagne. Ils ont été divisés en deux groupes : un groupe témoin et un groupe expérimental (*TAN*). La seule

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différence entre ces deux groupes était que le soja, incorporé comme supplément protéique dans le concentré de la ration *TAN*, avait été traité avec l'extrait de TH. Les quantités ingérées, l'efficacité alimentaire, le gain journalier et la durée de la période d'engraissement n'ont pas différé ($P > 0,10$) entre les deux groupes. L'examen histopathologique n'a montré aucun signe de toxicité dû aux tannins. De même, les carcasses du groupe *TAN* n'ont pas présenté de résidus de métabolites de TH (acide gallique, acide ellagique, réSORCINOL, PYROGALLOL et PHLOROGLUCINOL). Les activités enzymatiques de la gamma glutamyl transférase (GGT) et de l'aspartate amino-transférase (AST) élevées dans les agneaux du groupe *TAN* suggèrent que des expériences de plus longue durée doivent être conduites pour vérifier les effets de toxicité dans ces conditions.

agneaux / période de finition / tannins hydrolysables

1. INTRODUCTION

Tannins are secondary plant compounds occurring in a large variety of forages. They are conventionally classified into two major groups: hydrolysable and condensed tannins. Hydrolysable tannins (HT) consist of a carbohydrate core with phenolic carboxylic acids bound by ester linkages [12, 16].

One of the key goals of protein nutrition research in ruminants is to optimise the efficiency of utilisation of dietary nitrogen to maximise growth and milk production per unit of nitrogen (N) consumed [1, 18]. In the presence of tannins in the rumen, plant proteins may be bound and protected from microbial degradation, but are released in the abomasum, enabling protein digestion and absorption of amino acids in the small intestine [23]. HT have been shown, through in sacco and in vitro trials, to decrease ruminal degradation of soya bean meal, in sheep, without detrimentally affecting its intestinal digestion [11]. However, the use of HT as a feed additive for improving the digestive utilisation of protein-rich feeds will have to confront the widely held view that HT are toxic at very low concentrations.

Hydrolysable tannins undergo hydrolysis in the rumen to lower molecular weight phenolics, which may be absorbed from the intestine and cause toxicity [14, 25]. Livestock intoxications with hydrolysable tannins have been reported in several articles [8, 21, 26, 28] and are characterised by anorexia, depression, rumen atony, hepatic and renal failure, ulcerations and severe gastro-

enteritis. The severity of the lesions is highly dependent on the dose [28] and the type of HT consumed; most tannin toxicosis is related to oak HT [8, 21, 26].

Nevertheless, HT-treated protein supplements would only act as complementary ingredients of non-treated protein supplements, being included in the diet only in the minimum amounts needed to meet the ruminants' requirements for rumen undegradable protein. Thus, the hypothesis proposed here was that the amount of HT used as an additive in the diet of finishing lambs would be too low to be toxic or detrimental for the animals.

The present work was carried out to study whether the consumption of a small amount of a chestnut hydrolysable tannin extract by finishing lambs, under intensive feeding conditions, might be toxic or negatively affect their performance. The aim of this study was to ensure the absence of negative effects of HT as a feed additive and not to investigate the beneficial use of these extracts in increasing the amount of rumen undegradable protein and hence improving animal production.

2. MATERIALS AND METHODS

2.1. Animals and diets

Fourteen male Merino lambs were used in this study. However, during the pre-experimental phase, one lamb on the *Control* treatment was removed from the experiment because it suffered persistent diarrhoea.

Table I. Ingredients and chemical composition of the concentrates.

	<i>Control</i>	<i>TAN</i>
Ingredients (g·kg ⁻¹ DM)		
Barley	730	700
Soya bean meal		
· control	130	-
· HT-treated	-	160*
Molasses	90	90
Bicarbonate	20	20
Vitamin-mineral supplement	30	30
Chemical composition (g·kg ⁻¹ DM)		
Dry matter (g·kg ⁻¹)	916	915
Crude protein	132	137
Neutral-detergent fibre	162	146
Ash	80	82
Gross energy (MJ·kg ⁻¹ DM)	14.2	14.1

* Including 130 g chestnut HT extract per kg DM soya bean meal.

The 13 remaining lambs were finished, from 15 to 25 kg of live weight (LW). Twenty-five kg LW is the most common slaughter weight for most Spanish breeds [5].

Previously, the lambs had remained stalled with their mothers and were given free access to a commercial starter concentrate and alfalfa hay until the commencement of the trial (when they weighed approximately 15 kg LW). The lambs were treated with Vitasel (Lab. Ovejero, Spain) immediately after birth to avoid white muscle disease and later on with Miloxan (Merial Lab., Spain) to prevent enterotoxemias and with albendazol 2.5% Ganadexil® (Industrial Veterinaria, Spain) to control parasites.

This experiment was performed according to the Spanish Council for Scientific Research (CSIC) guidelines on the principles and ethical considerations that must be taken into account when using animals in research.

The 13 animals were randomly divided into two groups: one group was used as the control (*Control* group, $n = 6$) and the other one received the treatment with HT (*TAN* group, $n = 7$). Experimental conditions (individual pens, free access to clean water, etc.) were exactly the same for all the animals. The only difference between the groups was that the soya bean meal incorporated as the protein supplement in the concentrate offered to group *TAN* had been treated with 13% of a hydrolysable tannin extract (Tanino Vinitanon, Agrovín, S.A., Spain), that is commercialised by the wine-making industry. This HT extract (tannin content = 750 g·kg⁻¹, data provided by the company Agrovín, S.A.; dry matter (DM) = 920 g·kg⁻¹; organic matter (OM) = 985 g·kg⁻¹ DM) is extracted from chestnut trees and is a complex mixture, including both gallotannins and ellagitannins.

The *TAN* concentrate contained 20.8 g chestnut HT extract per kg DM. Ingredients and chemical composition of the two concentrates are shown in Table I.

Barley straw and concentrates were offered ad libitum to the lambs. The amount of feed offered was adjusted daily on the basis of the previous day's intake, allowing refusals of 15–20%. These were removed, weighed and dried daily to determine voluntary DM intake throughout the experiment.

Four further ruminally cannulated ewes (mean LW 47 kg; SE 0.8) that had never consumed tannins previously, were used to determine the in situ extent of DM and N degradation in the rumen [20]. Each sheep was offered 600 g of barley grain and 200 g of barley straw per day in two equal meals.

2.2. Procedures

Soya bean meal was sprayed with 350 mL water per kg containing 430 g·L⁻¹ of HT extract, dried at room temperature for about 4 days, and mixed with the other ingredients of the concentrate.

The in situ extent of DM and N degradation in the rumen of the two concentrates was determined using nylon bags (120 × 85 mm; Maissa[®], Spain). Five grams of each concentrate, ground to pass a 2-mm screen, were incubated in the rumen of each ewe for 3, 6, 12, 24 and 48 hours. The bags were introduced in the rumen before the morning feeding. After removal from the rumen, the bags were washed with cold tap water, and frozen (−30 °C) for 24 h to help to remove microbial attachment to feed particles. Once defrosted, the bags were washed with cold water in a commercial washing machine, dried in a force-air oven at 40 °C and weighed to determine DM losses. Nitrogen (N) concentration in the residues was measured to determine N disappearance. Zero-time losses were estimated by washing two bags per sample with the same washing programme used to wash the bags after the incubations.

Experimental lambs were weighed twice per week until they reached 23 kg and then every day up to 25 kg of live weight.

In order to detect early signs of toxicity, the blood samples were collected weekly from each animal from the moment they were individually penned (first sampling) until slaughter (last sampling). The samples were collected, before the morning administration of the feed, from the jugular vein into heparinised evacuated tubes (Ven-oject[®], Belgium) for subsequent plasma biochemical analysis.

All lambs were euthanised with an intravenous injection of barbiturate (Eutalender[®], Normón, Spain) when they had reached 25 kg of LW. The bodies were divided into two components: carcass and “non-carcass” and all portions (blood, skin, fat depots, parts of the gastrointestinal tract, etc.) were weighed individually. The gastrointestinal tracts were emptied and their contents were weighed to obtain the empty body weight (EBW). Carcasses were weighed twice: first immediately after slaughter and then again after 24 h of chilling at 4 °C. The dressing proportion was

calculated as the cold carcass weight expressed as a proportion of the slaughter weight. Each fraction, carcass and “non-carcass”, was cut into pieces, minced successively, mixed thoroughly and analysed for chemical composition.

The samples for pathological studies were collected from the liver, kidneys, and several areas of the digestive tract (oesophagus, reticulum, rumen, omasum, abomasum, small (duodenum, jejunum and ileum) and large (caecum, colon and rectum) intestines). Gross and histopathological examination was focused on the presence of lesions associated with tannin toxicosis, characterised by congestion, erosions and necrosis in the digestive tract mucosa together with liver and renal tubular cell degenerative changes [26, 28].

The carcasses of the animals from the *TAN* group were analysed to detect major residual metabolites of HT degradation (gallic acid, ellagic acid, resorcinol, pyrogallol and phloroglucinol).

2.3. Chemical analyses

The feeds offered were dried in an oven at 100 °C to constant weight and then analysed for Kjeldahl N, ash and gross energy by procedures of the Association of Official Analytical Chemists [2] and for neutral-detergent fibre according to the Goering and Van Soest technique [10]. Incubation residues were analysed for DM and N.

Carcass and “non-carcass” samples, after having been freeze-dried, were analysed for crude protein (N × 6.25), fat and ash, following AOAC procedures [2].

Plasma samples were assayed for activities of alkaline phosphatase (ALP; EC 3.1.3.1), gamma glutamyl-transferase (GGT; EC 2.3.2.2) and aspartate amino-transferase (AST; EC 2.6.1.1) enzymes and concentrations of creatinine and glucose, using commercially available kits (autoanalyser Hitachi 704, Japan).

Tissues for histo-pathological examination were fixed in 10% neutral buffered

formalin and dehydrated through graded alcohols before being embedded in paraffin wax. Several 4 µm thick sections were cut from each sample and stained with haematoxylin and eosin. Samples from the digestive tract were also stained with the Alcian blue-periodic acid-Schiff technique.

Residual metabolites of HT degradation (gallic acid, ellagic acid, resorcinol, pyrogallol and phloroglucinol) were analysed with a Waters liquid chromatograph (Model 600E, Milford, MA, USA) with a Waters model 484 UV, using a column Nova-Pack® C18 3.9 × 150 mm, 4 µm, following the method described by Murdiati et al. [17]. All standards were acquired from Sigma®. Phenolic metabolites were extracted from the carcasses according to Murdiati et al. [17].

2.4. Calculations and statistical analysis

Dry matter and nitrogen disappearances were fitted to the model described by Ørskov and McDonald [19] [$d = a + b(1 - e^{(-c \times t)})$], where d represents the loss from the bag after t hours, a the fraction that immediately disappears from the bag (intercept), b the fraction that is potentially degraded over time and c the rate of degradation of fraction b . The equation was fitted to in situ degradation profiles using the NLIN procedure of SAS [22]. The extent of rumen degradation (Dg) was estimated by using the parameters a , b and c and a ruminal passage rate (k_p) value of 0.0625, according to the equation described by Ørskov and McDonald [19]: $Dg = a + (b \times c / (c + k_p))$.

Data were subjected to analysis of variance using the GLM procedure of the SAS package [22]. The data related to voluntary intake and plasma biochemistry were analysed using a *split-plot* design with the tannin treatment (*TAN* vs. *Control*) as the main *plot* and weeks as the *subplot*. The animals were nested within the *TAN* and *Control* groups and used as the error term to contrast the tannin treatment effect. The residual

error was used to assess the effect of the week and its interaction with the tannin treatment.

3. RESULTS

The HT-containing concentrate presented a lower value for the extent of degradation in the rumen of the protein (0.669 vs. 0.764 g degraded per g ingested; $P < 0.05$), which was due to a significant reduction of both the immediately degradable fraction (a) and the rate of degradation (c). Significant differences were also observed for b -values and for DM a -value ($P > 0.01$). However, the DM extent of rumen degradation did not show significant differences between treatments (Tab. II).

Figure 1 shows the weekly evolution of daily concentrate intake in lambs from the *Control* and *TAN* treatment groups. There were no significant differences due to the treatment with the HT extract ($P > 0.10$; RSD = 119.9), nor were there significant differences in voluntary barley straw intake between the groups (47 vs. 66 g per day for the *Control* and *TAN* treatments, respectively; $P > 0.10$). High individual variability in the straw intake was observed (RSD = 24.4).

No significant differences ($P > 0.10$; Tab. III) were found in any of the following parameters: LW daily gain, feed conversion, length of the finishing period, cold carcass weight, losses from chilling and dressing proportion.

Individual weights of the different portions (blood, skin, fat depots, parts of the gastrointestinal tract, etc.) were observed to be similar in the two groups ($P > 0.10$). Chemical composition and energy content of the empty body weight did not show differences between the treatments either (Tab. IV).

The histo-pathological study showed no signs of toxicity due to tannins. This was in line with all previous results: there was the absence of pathological changes associated

Table II. Dry matter (DM) and nitrogen (N) rumen degradation parameters (*a*, *b* and *c* values) and the extent of rumen degradation of the *Control* and *TAN* concentrates.

	<i>Control</i>	<i>TAN</i>	SE
<i>a</i> -value (g per g incubated)			
DM	0.502 ^a	0.470 ^b	0.0021
N	0.310 ^a	0.217 ^b	0.0028
<i>b</i> -value (g per g incubated)			
DM	0.377 ^b	0.407 ^a	0.0011
N	0.635 ^b	0.719 ^a	0.0045
<i>c</i> -value (h ⁻¹)			
DM	0.212	0.260	0.0146
N	0.157 ^a	0.116 ^b	0.0035
Extent of rumen degradation (g degraded per g ingested; <i>k_p</i> = 0.0625)			
DM	0.794	0.797	0.0021
N	0.764 ^a	0.669 ^b	0.0122

SE: standard error; ^{a, b} means in a row with different superscripts differ significantly ($P < 0.05$).

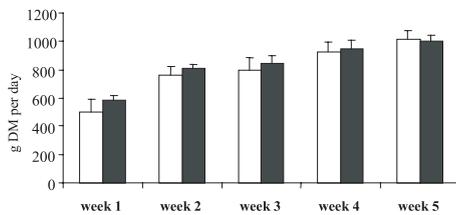


Figure 1. Weekly evolution of daily voluntary concentrate intake in lambs from *Control* (□) and *TAN* (■) groups. Vertical bars represent standard errors. There were neither significant differences between experimental groups, nor “treatment × week” interactions ($P > 0.10$). No significant differences were observed for straw intake either (mean values were 47 vs. 66 g per day for the *Control* and *TAN* treatments, respectively; $P > 0.10$).

with tannin intoxication in the gastric and intestinal mucosa, where no erosions or ulcers were seen, and in the liver and kidney which did not present any signs of parenchymal degeneration.

No differences between the animals in the *Control* or *TAN* treatment groups were observed prior to the beginning of the study for any plasma biochemical parameter (mean values on day 0 were 502 vs.

377 U·L⁻¹ for ALP, 57 vs. 62 U·L⁻¹ for GGT, 64 vs. 63 U·L⁻¹ for AST, 0.78 vs. 0.72 mg·dL⁻¹ for creatinine and 93 vs. 89 mg·dL⁻¹ for glucose, for the *Control* and *TAN* groups, respectively). As shown in Table V, the activity of ALP and concentrations of creatinine and glucose did not present any significant difference between the experimental groups ($P > 0.10$). However, the activities of GGT and AST were significantly higher in animals consuming the tannin-treated diet ($P < 0.05$), although no significant “treatment × week” interactions were found.

The analysis of the carcasses did not detect any residues of main HT metabolites (gallic acid, ellagic acid, resorcinol, pyrogallol and phloroglucinol), in the animals having consumed hydrolysable tannins.

4. DISCUSSION

As previously stated, the aim of this study was to ensure the absence of toxicity or negative effects when using HT, and not to investigate their beneficial use as a feed additive. Both concentrates (*Control* and *TAN*) were therefore formulated to include

Table III. Live weight (LW) daily gain, feed conversion (feed:gain ratio), length of finishing period, cold carcass weight, losses from chilling and dressing proportion in lambs from the *Control* and *TAN* groups.

	<i>Control</i>	<i>TAN</i>	SE
LW daily gain (g·day ⁻¹)	272	250	17.1
Feed conversion (kg concentrate per Δ kg LW)	3.3	3.4	0.12
Length of finishing period (days)	40	42	2.6
Cold carcass weight (kg)	11.1	11.2	0.11
Losses from chilling (%)	2.1	2.1	0.12
Dressing proportion (%)	44.3	44.5	0.47

SE: standard error; no significant differences between the experimental groups were observed in any of these parameters ($P > 0.10$).

Table IV. Empty body chemical composition and energy content in lambs from the *Control* and *TAN* groups.

	<i>Control</i>	<i>TAN</i>	SE
Chemical composition (g·100 g ⁻¹ EBW)			
Water	63.9	63.5	0.61
Crude protein	15.9	15.9	0.27
Fat	14.5	14.8	0.80
Ash	3.3	3.4	0.13
Energy content (MJ·kg ⁻¹ EBW)	9.6	9.9	0.25

SE: standard error; EBW: empty body weight; no significant differences between the experimental groups were observed in any of these parameters ($P > 0.10$).

similar crude protein contents, slightly below the lambs' requirements to make sure that possible detrimental effects of HT would not be concealed by high levels of protein [15]. In a like manner, although the *TAN* concentrate showed a lower value for the extent of protein degradation in the rumen, this dissimilarity was not high enough to be reflected in differences in the lambs performances.

All animals ate very little barley straw, which is common for finishing lambs [9]. No effects of the tannin extract inclusion on

Table V. Adjusted mean values of plasma activities of alkaline phosphatase (ALP), gamma glutamyl-transferase (GGT) and aspartate aminotransferase (AST) enzymes and concentrations of creatinine and glucose, in lambs from the *Control* and *TAN* groups.

	<i>Control</i>	<i>TAN</i>	SE
Enzyme activities (U·L ⁻¹)			
ALP	536.0	459.2	48.77
GGT	54.0 ^b	66.4 ^a	5.95
AST	67.0 ^b	79.7 ^a	5.93
Metabolites concentrations (mg·dL ⁻¹)			
Creatinine	0.73	0.69	0.020
Glucose	88.3	96.4	5.31

SE: standard error; ^{a, b} means in a row with different superscripts differ significantly ($P < 0.05$); there were no significant interactions "treatment × sampling week" ($P > 0.10$).

voluntary concentrate intake were observed in this work. However, tannins have been widely reported to depress voluntary feed intake (VFI) [13, 24, 28], with the depression being attributed to various factors. Firstly, tannins are reported to reduce palatability as a consequence of their astringent reaction in the mouth [12]. Nevertheless, the high proportion of molasses included in

the concentrate to reduce the lambs' selection of ingredients (both in the *Control* and *TAN* diets) resulted in a highly palatable diet in our experiment. A second factor, which may lead to a reduced VFI, is the reduction of the passage of nutrients through the gastrointestinal tract. This was not likely to have been a factor in this work since when data on DM rumen disappearance were fitted to an exponential model, no differences in the rate of degradation were found. Other authors [27] suggest that tannins can cause rapid and dramatic reductions in feed intake, likely mediated by stimulation of the emetic system and development of conditioned aversions. However, this explanation is also inadequate for our results since, according to the results described in the previous section, the *TAN* group did not suffer acute tannin intoxication. Thus, presumably the lack of a reduction of VFI in animals consuming the *TAN* diet, in spite of the inclusion of HT extract in the concentrate is related to the low dose of HT administered, which was insufficient to exert any significant effect on intake [3, 13].

No significant effects of chestnut extract inclusion on animal performance were observed (Tabs. III and IV). This was in agreement with the above-mentioned lack of differences in feed intake, together with the similarity in chemical composition and subtle differences, between diets, in rumen non-degradable protein contribution. As designed, these subtle differences were insufficient to lead to differences in productive responses. This suggests that the amount of HT ingested by the lambs was not sufficient to cause negative effects on animal performance.

Most HT toxicoses are natural poisonings documented in cattle fed oak and the real levels of HT consumed are seldom reported. Experimental intoxications show significant differences in the effects of HT depending on the type (chemical structure, molecular weight, etc.) and dose of tannins

and the animal species that consume them [21, 28].

In this experiment, all lambs presented very high values of ALP, which is physiologically normal in growing animals [7]. The differences between groups ($P < 0.05$) were found in AST and GGT activities. AST values may be representative of an acute hepatic lesion. Nevertheless, although they were higher in the group that consumed HT, they fell within the physiological range in both treatments. On the contrary, all GGT values (from *Control* and *TAN* groups) were higher than those reported in the literature as normal values. In addition, there were no "treatment \times week" interactions in any case, and the increase in enzyme activities was not supported by hepatic signs of toxicity found in the histo-pathological study. This makes it difficult to draw any categorical conclusion about the possibility of slight liver damage caused by the HT (or their degradation metabolites) action.

As is well known, hydrolysable tannins undergo rumen microbial and acid hydrolysis with the release of simpler phenolics [14, 25]. The toxicity of HT results from the absorption of large amounts of the simple phenolic compounds derived from their degradation [17]. The phenomenon where HT and simple phenolics are absorbed when tannins cause injury and ulceration of the gut lining [28] was not observed in this trial. Here the gut lining remained histologically normal and intact.

No changes in creatinine concentrations were found, suggesting that damage to the kidneys did not occur. There were no changes in plasma glucose concentration either. Increases in the latter parameter would have been related to the fact that tannins protect protein from degradation in the rumen and subsequently enhance amino-acid absorption, with glucose being synthesised by gluconeogenesis from amino acids [4]. Given the concentration of protein offered in the diet, however, this change was not expected.

Histo-pathological tests showed none of the signs of toxicity associated to HT toxicity (hepatic and renal failure, severe gastroenteritis, ulcers, etc.) [8, 21, 25, 28], which is in line with previous results and related, once again, to the low amount of HT ingested by the animals. In a like manner, and although it is true that other metabolites may not have been identified by the HPLC method [6], no residues of the major HT metabolites were detected in the carcasses of the animals consuming tannins.

Thus, the small amount of hydrolysable tannins ingested by lambs fed the *TAN* concentrate (which, as previously mentioned, contained 20.8 g of the chestnut HT extract per kg DM) does not seem to be toxic for the animals and had no observable detrimental effects on their productive performance.

Nevertheless, the potential use of HT as a feed additive to protect protein from rumen degradation still requires further investigation, in spite of the results of the present work and those reported by Hervás et al. [11]. This is because the lack of detrimental effects on lamb performance has only, thus far, been tested in young animals (up to 25 kg LW, which is the most common slaughter weight for lambs in Spain). In addition the increases in AST and GGT activities might indicate the development of cumulative effects, especially in other lambs finished to greater weights and over longer periods. Furthermore, HT might be degraded by adapted ruminal micro-organisms [14] and this may reduce or even remove the effectiveness of HT treatment for improving digestive utilisation of protein-rich feeds [11].

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