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AN LPS-BASED METHOD TO STIMULATE THE INFLAMMATORY RESPONSE IN GROWING RABBITS

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Abstract: Reliable indicators are needed to study the relationship between the inflammatory response of the growing rabbit and breeding factors such as feeding practices. A lipopolysaccharide (LPS) stimulation of the inflammatory response is a valid model of bacterial infection in laboratory animals, but no data on the growing rabbit has yet been obtained. The aim of our study was to determine an adequate dose of LPS to inject in growing rabbits in order to elicit a measurable inflammatory response in terms of plasmatic TNF-α and rise in rectal temperature. Three trials were carried out in this study: 2 development trials, the first (n=18) testing 3 doses of LPS (2, 10, 50 µg/kg) on the plasmatic TNF-α concentration at 90 and 180 min post injection, and the second trial (n=36) testing 4 doses of LPS (50, 75, 100 and 150 µg/kg) on the TNF-α concentration 90 min post injection and the rectal temperature. The third trial was designed as an application of the method in a large number of animals (n=32) to study the effect of feed restriction and dietary increase in digestible fibre to starch ratio on the LPS inflammatory challenge response of growing rabbits. In development trials 1 and 2, animals had measurable TNF-α responses for doses higher than 10 µg/kg at 90 min post injection, with an increase in the number of responsive animals along with the dose. High variability was observed in TNF-α concentrations in responsive animals (coefficient of variation from 44 to 94%). Animals demonstrated an increase in rectal temperature for all doses injected in the range of 50-150 µg/kg from 90 min post injection with a peak at 180 min (Δ_{Tr}=1.9±0.7°C). Our observations led us to choose a dose of 100 µg/kg of LPS for our following studies, as the responses in terms of temperature and TNF-α were the most satisfactory. The application of our LPS injection protocol to our nutritional study enabled us to validate our protocol (Δ_{Tr}=1.1±0.7°C at 180 min and 15/32 TNF-α responsive animals) even though we were not able to demonstrate any effect of the feeding level or diet on the inflammatory response to an LPS injection.

Key Words: rabbit, immune response, LPS, inflammation, TNF-α.

INTRODUCTION

Classically, the immune response of a growing animal can be evaluated after stimulation of its immune system through specific antigen administrations (ovalbumin, lipopolysaccharides...) (Meissonnier *et al.*, 2008; Qiu *et al.*, 2013). Lipopolysaccharide (LPS) is a structural component of the cell wall of Gram-negative bacteria and a potent inducer of inflammatory response. It is widely recognised as a valid model of bacterial infection (Redl *et al.*, 1993), even though some authors contest its representativeness, as the inflammatory response can be significantly higher than in sepsis models (Granger *et al.*, 2006). Intra-venous (i.v.) and intra-peritoneal (i.p.) injections of LPS are known to cause fever and a release of pro-inflammatory cytokines. Among those, Tumour Necrosis Factor alpha (TNF-α) plays a key role as a mediator of inflammation and septic shock and is implicated in fever pathogenesis (Mabika and Laburn, 1999). Thus, the measurements of temperature and TNF-α levels can be considered adequate indicators of

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the inflammatory response to LPS injections in the adult rabbit (Huang *et al.*, 2008; Ferrian *et al.*, 2013). However, the dose used to elicit a measurable inflammatory response varies greatly according to the authors, from doses below 2 µg/kg (Shibata *et al.*, 2005; Huang *et al.*, 2008) to doses above 50 µg/kg (Brito *et al.*, 1995; Ferrian *et al.*, 2013).

Even though the use of LPS has been studied in adult rabbits, no data has yet been obtained in growing rabbits. The first step in our study was therefore to determine the appropriate dose of LPS to inject in growing rabbits in order to obtain a measurable inflammatory response in terms of plasmatic TNF-α and rise in rectal temperature. We also wanted to determine the adequate time of measurement of the plasmatic concentrations of TNF-α.

Young rabbits are at high risk of developing digestive disorders during the 2 first weeks following weaning. These disorders are often associated with inflammation of the whole or part of the digestive tract (Marlier *et al.*, 2003). Short term feed restriction is a well-known method to reduce digestive disorders in weanling rabbits. A reduction of more than 20% in the feed intake allows to reduce post-weaning mortality and morbidity by up to 50% (Gidenne *et al.*, 2012). Likewise, an increase in the dietary ratio of digestible fibres to starch decreases the incidence of digestive disorders in weanling rabbits (Perez *et al.*, 2000). These beneficial effects upon health could be associated with a decreased inflammatory response, as previously demonstrated in other species submitted to feed restriction (Matsuzaki *et al.*, 2001; MacDonald *et al.*, 2011a) or fed fibrous diets (Kuo, 2013). Thus, the second aim of our study was to determine whether the beneficial effects of feed restriction and a dietary increase in digestible fibre to starch ratio upon health were associated with a decreased inflammatory response to an LPS injection. Moreover, this study enabled us to validate this in vivo experiment on a wider scale.

MATERIALS AND METHODS

Three trials were carried out in this study: Trials 1 and 2 were set up to determine the appropriate dose of LPS to inject the rabbits with and the optimal method to measure the subsequent inflammatory response. Trial 3 was designed as an application of the method to study the effect of feed restriction and diets differing in nutritional values on the LPS inflammatory challenge response of growing rabbits.

Animals, housing and feeds

The three trials were conducted at the INRA UE PECTOUL (Castanet-Tolosan, France) breeding unit using healthy hybrid rabbits (*Oryctolagus cuniculus*). The rabbits were housed in collective cages of 3 to 6 animals (density: 10, 18 and 15 rabbits/m^2 respectively for trials 1, 2 and 3) in a closed unit where the environment (temperature, lighting and ventilation) was monitored and controlled.

The animals in trials 1 and 2 were fed a standard post-weaning diet without antibiotics *ad libitum* (Table 1). Animals in trial 3 were fed one of 2 experimental diets (ST, rich in starch *vs.* DF, rich in digestible fibres) according to their treatment. These experimental diets were formulated to meet the nutritional requirements for growing rabbit (De Blas and Mateos, 2010) without any drug supplementation (antibiotics or coccidiostatics) (Table 1). The feeds were manufactured and pelleted at the same time, using one batch of raw material from Euronutrition SAS (Saint-Symphorien, France). All animals had free access to water and were handled according to the recommendations in animal care in experimentation in accordance with French national legislation. The animals were weighed individually upon arrival, at 46 d of age in trial 1 and 35 d of age in trials 2 and 3, and before injection, at 52 d of age in trial 1 and 42 d of age in trials 2 and 3 (Figure 1). Health status was assessed by clinical examination of the animals before injection. This consisted of checking the animals for clinical signs of digestive disorders such as diarrhoea, caecal impaction, suspicion of ERE (Epizootic Rabbit Enteropathy) or other pathologies (respiratory problems, injuries…). Animals without clinical signs of illness but presenting weight losses or very low growth (3 standard deviations below the mean) were considered morbid and excluded from further analyses.

LPS preparation and administration

For all trials, lyophilised LPS (*Escherichia coli* O26:B6; Sigma-Aldrich, St. Quentin Fallavier, France) was dissolved in saline (0.9% NaCl) to working dilutions under sterile conditions. All rabbits then received an intra-peritoneal injection of 2.5 mL of solution per kg of live weight.

Table 1: Ingredients and formulated chemical composition of the experimental diets.

		Trial 3	
Ingredients (%)	Trials 1 and 2	ST	DF
Wheat	0.00	9.00	12.40
Barley	7.00	15.00	2.00
Wheat bran	28.54	3.70	5.40
Rapeseed	0.00	2.00	2.20
Rapeseed meal	2.40	2.80	9.40
Sunflower meal	26.20	25.00	21.80
Alfalfa	8.00	11.10	2.00
Wheat straw	0.00	2.80	6.50
Sugar beet pulp	16.60	12.90	25.00
Grape pulp	4.80	2.00	2.60
Apple pomace	0.00	6.30	5.30
Cane molasses	3.60	4.31	3.00
Rapeseed oil	0.30	0.50	0.50
Dicalcium phosphate	0.00	0.19	0.11
Calcium carbonate	1.01	0.00	0.00
Salt	0.65	0.00	0.00
Methionine 15%	0.00	0.30	0.15
L-Lysine 20%	0.00	0.75	0.55
Threonine 10%	0.00	0.35	0.09
Vitamin premix	0.90	1.00	1.00
Chemical composition (%)			
Crude protein	16.05	15.50	15.51
Starch	11.55	16.00	11.86
Cellulose	19.22	16.70	16.68
Crude fat	2.51	3.10	2.99
Digestible fibre[1]		17.00	22.00

[1] Calculated according to tables of ingredients (Maertens *et al.*, 2002). ST: diet rich in starch; DF: diet rich in digestible fibres.

Experimental treatments

Trial 1, development step one.

At 52 d of age, 18 rabbits were randomly allotted into 3 groups of 5 rabbits receiving different doses of LPS (2, 10 and 50 µg/kg) and a control group of 3 rabbits receiving a saline solution (0.9% NaCl). Special care was taken to ensure an equivalent mean weight of the rabbits between the groups. Trial 1 was the first step of our study that aimed to define an *in vivo* methodology. Given the result of trial 1, we hypothesised in trial 2 and 3 that an earlier administration of LPS would provide a more suitable response. Indeed, in growing rabbits, mortality is more likely to occur during the first weeks following weaning, when the sanitary risk is at its highest. We hypothesised that changes in the inflammatory response should also occur at a similar time, and changed the trial date to 42 d of age (1 wk after weaning).

Trial 2, development step 2.

At 42 d of age, 36 rabbits were randomly allotted into 6 groups of equivalent mean weight. The first two groups received either a saline injection or an injection of 100 µg/kg of LPS. As the rabbits demonstrated good recovery, 3 new doses were tested the following day (43 d of age). Thus, the 24 remaining rabbits were allotted into 4 groups of 6 animals receiving a saline injection or 50, 75 or 150 µg/kg of LPS.

Trial 3, application.

Three hundred and twenty animals were divided at weaning, at 35 d of age, into 4 groups differing in dietary energy source (ST, rich in starch *vs.* DF, rich in digestible fibres), and feeding level (*ad libitum* (100) or restricted at 75% (75))

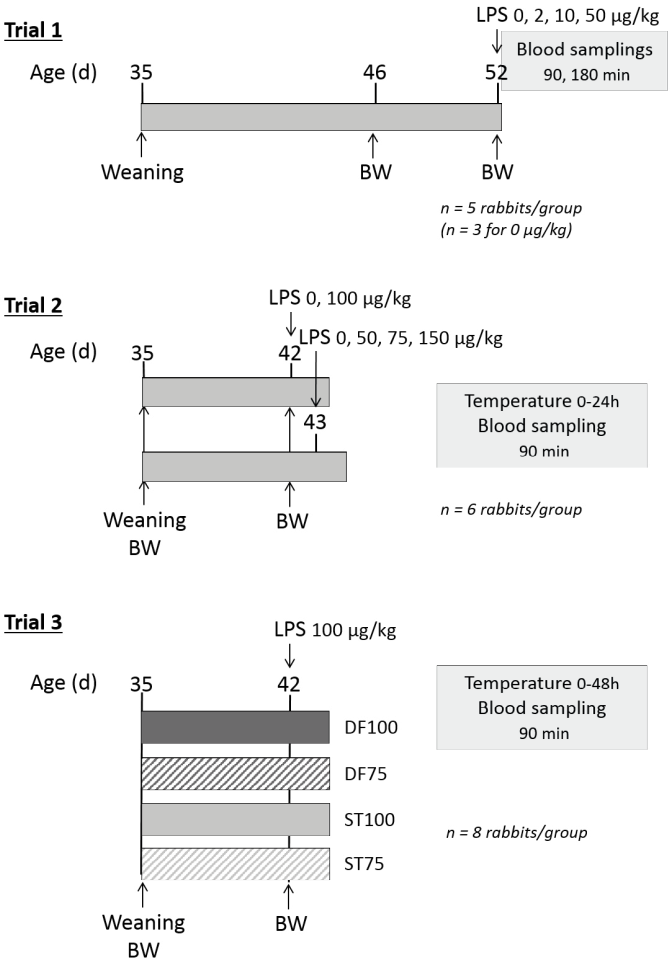

Figure 1: Experimental design of the 3 trials. ST: Diet rich in starch; DF: Diet rich in digestible fibres; 100: *Ad libitum* feeding; 75: Restricted feeding at 75% of the *ad libitum* intake.

in a 2×2 experimental design. The amounts of feed distributed to the restricted animals were calculated on the basis of a theoretical *ad libitum* ingestion curve, and readjusted for each diet according to the real ingestion of the *ad libitum* fed groups (ST100 and DF100). Feed was given in a single distribution each day between 8:00 and 8:30. At 43 d of age, 8 rabbits from each experimental treatment were selected and injected with 100 µg/kg of LPS. The average weight and standard deviation of the rabbits selected and those of the remaining animals were equivalent in order to have a representative sample of the experimental animals.

Rectal temperature measurements (trials 2 and 3)

Rectal temperature was recorded with a digital thermometer (MT-403, Hangzhou Sojoy electronics & instruments co., China) every 30 min from the time of injection until 180 min post-injection (3 h), then the temperature was recorded at 6 h and 24 h for all trials and 48 h (only for trial 3). The temperature recorded immediately prior to injection

was considered the baseline temperature and temperature changes were calculated as the difference between the recorded temperature at one time-point and the baseline.

Blood samplings

Blood from all the injected rabbits was retrieved from the marginal ear vein 90 min post injection. Blood was also collected from the aorta at euthanasia at 180 min after LPS injections in trial 1. Blood was collected in heparinised tubes (Vacuette, 9mL NH Sodium Heparin; Greiner Bio-One, Kremsmünster, Austria) and immediately transferred on ice until arrival at the laboratory. The tubes were then centrifuged at 1000 *g* for 10 min at 4°C. The plasmas were retrieved and stored at –20°C until further analysis.

Determination of plasma concentrations of TNF-α

Plasma TNF-α concentrations were quantified by enzyme-linked immunosorbent assay (ELISA) using specific anti-rabbit TNF-α antibodies (Rabbit TNF-α DuoSet, R&D Systems, Abingdon, UK) following the manufacturer's recommendations. Briefly, flat-bottom 96-well microtitre plates were coated with 100 µL/well of polyclonal mouse anti-rabbit TNF-α antibody diluted at 2 µg/mL in coating buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.2-7.4) and incubated overnight at room temperature. Plates were washed 3 times with PBS-Tween and blocked with 200µL/well of reagent diluent (1% BSA in PBS) for 1 h at room temperature. Plates were then washed again 3 times and standard and samples were added, followed by 2 h of incubation at room temperature. Samples were analysed in triplicate, appropriately diluted in reagent diluents. Plates were washed 3 times, followed by the addition of 100 µL/well of biotinylated goat anti-rabbit TNF-α antibody diluted at 100 ng/mL in reagent diluent. After 2 h of incubation at room temperature, the plates were washed again 3 times and 100 µL of Streptavidin-HRP, diluted at 1:200 in reagent diluent, was added to each well. The plates were incubated for 20 min at room temperature in the dark. The plates were then washed 4 times and 100 µL of substrate solution (1:1 H_2O_2 - Tetramethylbenzidine) (Thermo Fisher Scientific) was added to each well. The plates were then incubated for 20 min in the dark until the desired coloration was obtained, which was followed by the addition of 50 µL/well of stop solution (2 N H_2SO_4). Optical density (OD) of each well was read at 540 nm and subtracted from the readings at 450 nm (Spectra thermo scan, Tecan) to correct for the optical imperfections in the plates. The mean OD of each sample was then calculated, and the mean value of the negative control was subtracted from all sample values. Sample TNF-α concentrations were then obtained, thanks to the standard curve elaborated with the OD values of the diluted recombinant rabbit TNF-α standard of known concentration.

Data Analysis

Temperature measurements were analysed using the MIXED procedure (SAS) with the dose and time as fixed effects in trial 2, and the feed intake level, diet, time and interaction between intake level and diet as fixed effects in trial 3, whereas the rabbit was set as random effect. Mean comparisons were performed using the Bonferroni test. Weight, growth and TNF-α concentrations in trial 3 were compared using the MIXED procedure with the intake level, diet and interaction between the intake level and diet as fixed effects. Finally, the occurrence of TNF-α responsive animals was analysed using a Chi-squared test. Pearson's correlation was performed to link TNF-α concentrations at 90 min post injection and maximal rectal temperature changes from baseline.

RESULTS

Growth measurements

Among the different treatments, the animals had similar growth and live weight prior to the injections in trials 1 and 2 (respectively 39.1±19.4 g/d and 1613±147 g at 52 d of age and 63.6±7.4 g/d and 1523±77 g at 42 d of age). In trial 3, the LPS injected animals submitted to feed restriction had a growth reduced by 44% and a weight at 42 d of age reduced by 12% compared to the animals fed *ad libitum* (Table 2). The diet (ST *vs*. DF) did not affect the growth and weight of the animals. Two animals in trial 1 (in the groups injected with 2 and 10 µg/kg) were excluded

Table 2: Effect of diet and feeding level on growth and plasmatic concentrations of TNF-α 90 min after a 100 µg/kg LPS injection in trial 3 (n=8 rabbits per group).

	Group[1]				*P*-values		
	ST100	ST75	DF100	DF75	Diet	Intake level	Diet×level
Weight at 35 d of age (g)	1055±112	1041±122	1044±144	1036±131	NS	NS	NS
Weight at 42 d of age (g)	1419±136	1262±122	1414±168	1223±115	NS	0.001	NS
Daily Weight Gain (g)	51.9±7.7	31.5±4.3	52.9±6.3	26.8±7.3	NS	<0.001	NS
TNF-α n/ni[2]	4/8	3/8	3/8	5/8	NS	NS	NS
TNF-α (ng/mL)	1.64±1.69	3.47±4.16	0.42±0.01	4.32±5.35	NS	NS	NS

ST: Diet rich in starch; DF: Diet rich in digestible fibres; 100: *Ad libitum* feeding; 75: Restricted feeding at 75% of the *ad libitum* intake. NS: not significant.

[1]Values are presented as means±sd.

[2]Number of animals presenting detectable concentrations of TNF-α on the total number of animals.

from the analysis, as they manifested weight losses prior to injection. None of the other animals used for the 3 trials presented clinical signs of illness. No mortality was observed 48 h post LPS injection (trials 2 and 3).

Effect of LPS injection on rectal temperature

The rabbits had an average basal temperature of 38.9±0.4°C (trials 2 and 3). In trial 2, fever was observed for all doses injected 90 min after injection. The fever was observed at earlier stages with the highest doses (at 30 min for 100 µg/kg and 60 min for 150 µg/kg) (Figure 2 A, B). From 90 min to 360 min post injection, there were no significant differences in fever levels between the different doses. The temperature rose for all groups until 180 min (Δ=1.9±0.7°C on av. for all groups corrected for their corresponding controls) and gradually decreased afterwards (Figure 2 C). After 24 h there was no difference between the control groups and the groups injected with 50 and 100 µg/kg (Figure 2 A, B).

In trial 3 there was no effect of the diet or the feeding level on the rectal temperature at any time-point in the animals injected with 100 µg/kg of LPS (Figure 3). However, for all the groups, as in trial 2, a fever was observed from 60 min post injection with a peak at 180 min (Δ=1.1±0.7°C). The temperature was back to basal levels after 48 h for all groups.

Effect of the LPS injection on plasmatic TNF-α concentrations

In trials 1 and 2, throughout the experimental period none of the control animals presented detectable concentrations of TNF-α. Concerning the animals injected with LPS, depending on the dose, a variable proportion of the animals injected with LPS presented a detectable concentration of TNF-α 90 min after the injections (Table 3). 180 min after injection, only one animal in the group injected with 50 µg/kg still presented a measurable concentration of plasmatic TNF-α (900 pg/mL, data not shown). Even though the number of animals responsive to the injection was numerically higher in the groups injected with 100 µg/kg and 150 µg/kg, there was no significant effect of the dose

Table 3: Effect of the LPS dose injected on the plasmatic concentrations of TNF-α 90 min after injection in trials 1 and 2.

	Group						
	Controls[1]	2 µg/kg	10 µg/kg	50 µg/kg[1]	75 µg/kg	100 µg/kg	150 µg/kg
n/ni[2]	0/15	0/5	1/4	4/11	1/6	5/6	6/6
TNF-α (ng/mL)[3]	ND	ND	8.23	4.38±4.10	2.34	4.21±1.86	5.04±2.90

[1]Animals from trials 1 and 2 combined.

[2]Number of animals presenting detectable concentrations of TNF-α on the total number of animals.

[3]Values are presented as means±sd.

ND = Not detectable.

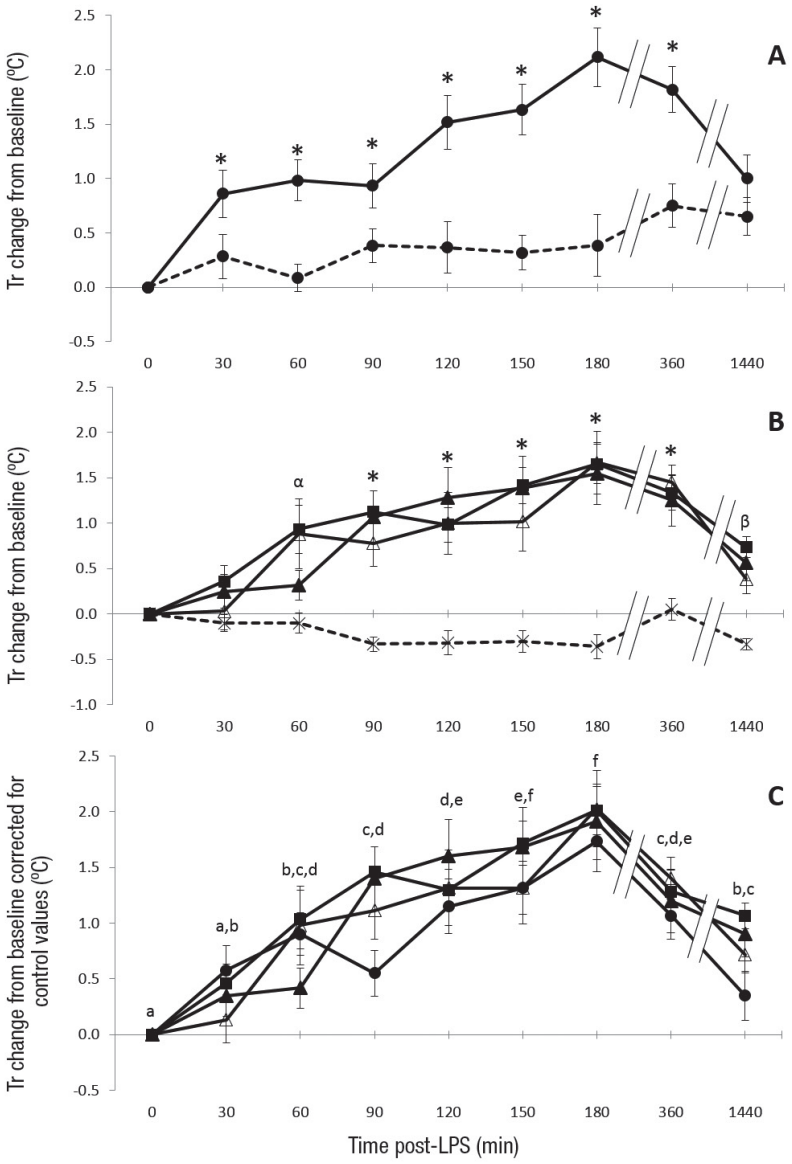

Figure 2: Effect of the LPS dose injected on the mean rectal temperature change from baseline in trial 2 (n=6 rabbits per group). Tr: Rectal temperature. Values are presented as means±standard error of the mean. A and B: *P<0.05, tested doses are significantly different from corresponding control value for a given time-point (control 1 for dose 100 µg/kg and control 2 for dose 50, 75 and 150 µg/kg); B: α P<0.05, significant difference between dose 15 µg/kg and control 60 min post injection; β P<0.05, significant difference between doses 75 and 150 µg/kg and control 24 h (1440 min) post-injection. C: a,b,c,d, e, f : mean values of all measured rabbits at different time-points without a common superscript differ at the level of 0.05. A: --●-- Control 1; —●— 100 µg/kg. B: --×-- Control 2; —△— 50 µg/kg; —▲— 75 µg/kg; —■— 150 µg/kg. C: —△— 50 µg/kg; —▲— 75 µg/kg; —●— 100 µg/kg; —■— 150 µg/kg.

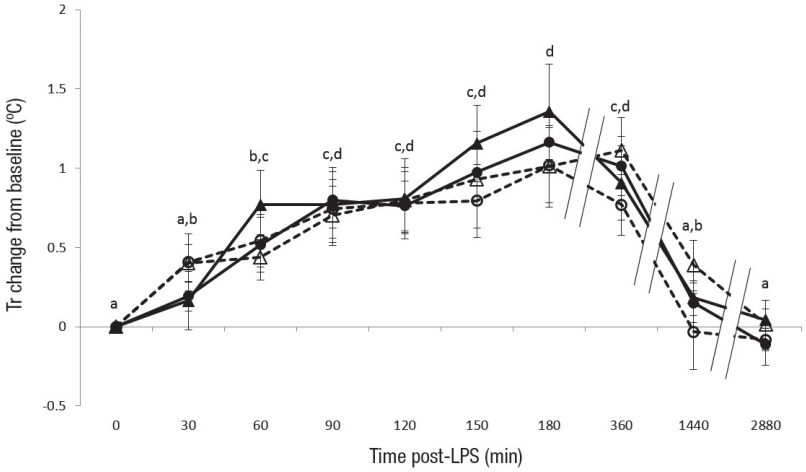

Figure 3: Effect of diet and feeding level on the mean rectal temperature change from baseline in animals injected with 100 µg/kg of LPS in trial 3 (n=8 rabbits per group). Tr: Rectal temperature. Values are presented as means±standard error of the mean. ST: Diet rich in starch; DF: Diet rich in digestible fibres; 100: *Ad libitum* feeding; 75: Restricted feeding at 75% of *ad libitum* intake. a,b,c,d : mean values of all measured rabbits at different time-points without a common superscript differ at the level of 0.05. –●– ST100; --○-- ST75; –▲– DF100; --△-- DF75.

upon the number of responsive animals. Moreover, we observed a great variability of the TNF-α concentrations in the responsive animals (CV from 44 to 94%). Thus, no significant effect of the dose was observed on the TNF-α concentrations.

In trial 3, there was no significant effect of the diet or the feeding level upon the TNF-α response to an injection of 100 µg/kg of LPS (Table 2). As in the development trials (trials 1 and 2), we observed a great variability of the TNF-α concentrations in the responsive animals (CV from 2% to 124%). Moreover, in both trials 2 and 3, no correlation was observed between the maximum elevation of temperature and the concentration of TNF-α measured in the responsive animals 90 min post injection (r^2=0.074) (Figure 4).

DISCUSSION

LPS is a well-known and recognised inducer of inflammatory response in laboratory animals (Redl *et al.*, 1993). However, all studies to date in rabbits use small groups of adult individuals. Our study is therefore the first to adapt an LPS injection protocol to the growing rabbit.

Consistent fever in response to LPS injections

None of the animals used for all 3 trials presented clinical signs of illness. Thus, we could assume that the inflammatory reactions measured would be caused by the LPS injections. In the second development trial and the application trial, all animals injected with LPS presented an elevation of temperature compared to baseline and control groups, thus demonstrating a fever in response to the LPS injections. The fever was at its highest at 3 h post injection, confirming results obtained in male adult rabbits (Mabika and Laburn, 1999; Shibata *et al.*, 2005; Huang *et al.*, 2008) where the fever was at its highest between 180 and 200 min post injection. In the application trial, the temperature for all groups was back to baseline 48 h post-injection, as observed in adult rabbits with a dose of 50 µg/kg (Ferrian *et al.*, 2013). Few differences in fever were noted for doses in the range 50-150 µg/kg and no mortalities were observed in the 48 h following the injections. This was quite surprising, as mortality has been reported in adult animals for doses higher than 100 µg/kg with a rapid increase of the occurrences along with the dose (Brito *et al.*, 1995). A 42% mortality rate was even observed at 48 h post-injection for a dose of 50 µg/kg in lactating does (Ferrian *et al.*, 2013). However,

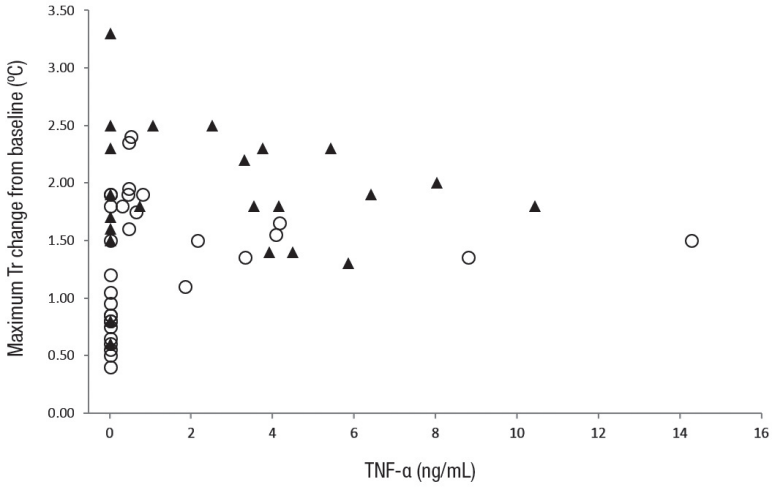

Figure 4: Correlation between maximal rectal temperature changes from baseline and TNF-α concentrations at 90 min post injection. Tr: Rectal temperature. r^2=0.074, was calculated on the animals responsive in terms of TNF-α concentrations only. ▲ Trial 2; ○ Trial 3.

equivalent rises in temperatures were found in our experiments and in the studies cited above. Thus, the reduced mortality in our study is not related to a reduced fever in growing rabbits. Fever is not the only element that triggers mortality, so we could therefore hypothesise that the growing rabbit is more resistant to LPS-induced inflammation even though the fever is not reduced.

A highly variable TNF-α response to LPS injections

In the development studies, animals only had measurable TNF-α responses for LPS doses higher than 10 µg/kg. This was surprising, as measurable concentrations of TNF-α were reported for adult individuals for LPS doses lower than 2 µg/kg (Shibata *et al.*, 2005; Huang *et al.*, 2008). This might be due to the injection route chosen or a response of the animals prior to the time of measurement. However, the highest TNF-α concentrations in adult rabbits have been observed between 60 to 120 min post-LPS injection (Brito *et al.*, 1995; Shibata *et al.*, 2005; Huang *et al.*, 2008), thus confirming the accuracy of our measurement time. Unlike previous studies in adult rabbits using i.v. injections, an i.p. route was used in our study for LPS administration, as it has been demonstrated in mice and rats that the i.p. route is more adequate to mimic sepsis, inducing stable plasmatic levels of LPS for 2 to 5 h with an increased mortality and cytokine production (Redl *et al.*, 1993; Remick, 2004). Thus, our measured concentrations of TNF-α should have been higher than those previously observed by authors using i.v. injections. A hypothesis could be that the rabbit does not react like the mouse or the rat to the injection route and that the i.v. route would be favourable to trigger an inflammatory reaction in rabbits. Also, as published by Dinges and Schlievert (2001), an inoculation of TSST-1 (Toxic Shock Syndrome Toxin 1) prior to the LPS inoculation could increase the production of plasmatic TNF-α in response to LPS. Thus, for future studies, this co-inoculation could be considered as an alternative method.

In both development trials, the responsive animals had very variable responses to the LPS injections in terms of TNF-α concentrations. Other authors have observed a similar high variability in the concentrations of TNF-α (CV of 15 to 100%) in adult rabbits (Brito *et al.*, 1995; Huang *et al.*, 2008) thus indicating a high individual TNF-α specificity. Despite the important variability in responses, the number of reactive animals grew with the elevation of the injected dose as observed by Brito *et al.* (1995) in adult animals.

Interestingly, in the second development trial and the application trial the highest TNF-α concentrations were not correlated with the highest increases in body temperature. Likewise, animals without detectable concentrations of TNF-α had fever. This result did not agree with most studies, which show a positive correlation between fever and

TNF-α levels, indicating TNF-α as a pyrogen (Mabika and Laburn, 1999). The absence of correlation between fever and TNF-α levels in our study might be associated with the high variability in TNF-α responses. However, some studies in rats have suggested a potential cryogenic action of TNF-α, with a reduced TNF-α response to LPS during heat stress (Kluger *et al.*, 1997) or an increased fever when animals were injected with antiserum against TNF-α (Long *et al.*, 1990). Accordingly, the correlation between TNF-α and fever might be more complex than previously expected.

A valid method in a large scale experiment

Our observations led us to choose a high dose of LPS for further studies in order to elicit satisfactory responses in terms of rectal temperature increase or TNF-α concentrations. As similar results were obtained for the doses 100 and 150 µg/kg, we chose to use the lowest dose (100 µg/kg) for our follow-up studies. First, from an ethical point of view, the lowest efficient dose appeared to be the most reasonable. Secondly, Feuerstein *et al.* (1990) showed that the TNF-α response to LPS reached a plateau in rats for a dose higher than 100 µg/kg. Even though adult rats are known to be less sensitive to LPS than adult rabbits (Redl *et al.*, 1993), our observations in growing rabbits were coherent with Feuerstein's observations, thus leading us to choose the 100 µg/kg dose. Finally, our observations in the first development study led us to measure TNF-α concentrations at 90 min post injection.

The application of our LPS injection protocol to our nutritional study enabled us to obtain results that were consistent with those achieved in our development trials, thus allowing us to validate our protocol. However we were surprised not to find any effect of feed restriction upon the inflammatory response to LPS. In mice and rats it has been demonstrated that a 4 wk feed restriction period reduced the inflammatory response to LPS through a reduced fever period and intensity (MacDonald *et al.*, 2011b; MacDonald *et al.*, 2012) and a reduced concentration of TNF-α (Matsuzaki *et al.*, 2001). However, in our study, animals were only feed restricted for 1 wk, which might not be a sufficiently long period to induce a significant effect on the inflammatory response. The diet did not affect the inflammatory response to the LPS injection. Accordingly, the reduced incidence of digestive disorders observed with a higher ratio of digestible fibres to starch (Perez *et al.*, 2000) would not be related to a modulation of the inflammatory status. However, Pie *et al.* (2007) demonstrated an increased inflammatory response in weaning piglets fed a diet supplemented with fermentable carbohydrates. Despite the limited available information on the possible effects of the nutrient intake on the inflammatory response, the short period of time for which the animals were exposed to their new diet could also explain the lack of effect of the diets on the inflammatory response.

CONCLUSION

This study enabled us to draw up an LPS injection protocol for the growing rabbit, validated in a large number of animals, using a 100 µg/kg dose of LPS and measurements of rectal temperatures and TNF-α concentrations. Rectal temperature appears to be a simple and reliable criterion to evaluate the inflammatory status in a large number of rabbits, whereas measurements of TNF-α concentrations remain highly variable and their interpretation can be difficult. Moreover, our study suggested that inflammatory response in the growing rabbit follows the same pattern as in the adult, even though the growing rabbit appears to have a higher resistance to LPS-induced inflammation.

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