Quantitative Feed Restriction Rather Than Caloric Restriction Modulates the Immune Response of Growing Rabbits1–3

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Abstract

Background: Short-term feed restriction strategies are used in rabbits to reduce postweaning digestive disorders, but little is known about the involvement of the immune system in these beneficial effects.

Objective: In the present study, the consequences of feed and energy restriction on immune response were investigated.

Methods: At weaning, 320 male and female rabbits were assigned to 4 groups differing in dietary digestible energy (DE) concentrations and intake levels: a low-energy ad libitum–feed (LE100) group, a low-energy restricted-feed (LE75) group, a high-energy ad libitum–feed (HE100) group, and a high-energy restricted-feed (HE75) group. The high-energy groups consumed 10.13 MJ DE/kg of feed, whereas the low-energy groups consumed 9.08 MJ DE/kg (formulated values). Intake amounts for the restricted groups were 75% those of the ad libitum groups. Rabbits consumed these diets until age 63 d, after which they consumed feed ad libitum for 9 d. Ten rabbits per group and per age were killed at ages 42, 50, 63, and 72 d.

Results: The relative weight and size of the lymphoid organs were not affected by treatments. Concentrations of plasma total immunoglobulin (Ig) G and anti-ovalbumin IgG; and fecal and plasma IgA concentrations were determined by ELISA; and ileal expressions of cytokines were measured by quantitative reverse transcriptase-polymerase chain reaction at ages 50 and 63 d.

Conclusion: These results demonstrated that, in rabbits, restriction and, to a lesser extent, dietary energy concentration modulate gut immunity.

Keywords: rabbit, feed restriction, energy intake, health, immune response

Introduction

Severe dietary changes often cause digestive disorders. In mammals, weaning generates important changes as the young animal shifts from milk to solid feed, and is often responsible for various digestive disorders (1, 2). These disorders are particularly important in agricultural species because they result in economic losses for the breeder. Moreover, current European legislation and recommendations strongly discourage the use of antibiotics (3); thus, new alternatives are needed to preserve animals’ health (4). Short-term feed restriction was proven to reduce postweaning digestive disorders in rabbits and has been used by breeders in France for over 10 y as an efficient method to preserve the digestive health of growing rabbits (5). Indeed, a reduction in feed intake (below 20% of free intake) reduces postweaning mortality and morbidity (6). Previous studies in mice and rats suggested

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3 Supplemental Tables 1–3 and Supplemental Figure 1 are available from the “Online supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

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that long-term feed restriction and the nutritional value of the diet could affect gut immunity (7–9). In growing rabbits, the effect of some nutrients, such as starch and fiber, on the immune response was documented. Indeed, increased intake of fiber could improve mucosal immunity through an increased number of membranous epithelial cells in the appendix of postweaning rabbits (10). However, the involvement of the immune system in the reduction in postweaning digestive troubles through short-term feed restriction is poorly documented. Likewise, to our knowledge, the short-term effect of dietary energy concentration on the immune system has not yet been studied in agricultural species. Thus, the present study was conducted to evaluate the impact of both quantitative feed restriction and dietary digestible energy (DE)\textsuperscript{15} concentration on gut immunity in growing rabbits.

### Methods

#### Study design, rabbits, housing, and feed.

The study was conducted at the INRA (Institut National de la Recherche Agronomique) UE (Unité Expérimentale) PECTOUL (Pôle Éxperimental Canicole de Toulouse) breeding unit in Castanet-Tolosan, France, on 320 healthy male and female hybrid rabbits (*Oryctolagus cuniculus*) at a 50:50 male-to-female ratio. A bifactorial design was used with 2 levels of feed intake—ad libitum feed consumption vs. restricted feeding at 75\% of ad libitum—and 2 diets differing in DE concentration—a low-energy (LE) diet, formulated with 9.08 MJ DE/kg, and a high-energy (HE) diet, formulated with 10.13 MJ DE/kg according to the European Group on Rabbit Nutrition tables from Maertens et al. (11). The rabbits were assigned to 4 treatment groups: a low-energy ad libitum–feed (LE100) group, a low-energy restricted-feed (LE75) group, a high-energy ad libitum–feed (HE100) group, and a high-energy restricted-feed (HE75) group. The diets were formulated to meet the nutritional requirements of growing rabbits (12), without drug supplementation (antibiotics or coccidiostats). Special attention was given to obtaining a theoretical deviation of 1 MJ/kg of DE between the diets, with the constraint of obtaining similar ratios of digestible fibers, starch, and fat to DE, starch to digestible fibers, and digestible proteins to DE. Likewise, both diets were formulated with equivalent concentrations of acid detergent fiber and neutral detergent fiber (*Supplemental Table 1*).

The feeds were manufactured and pelleted with the use of one batch of raw materials by Euronutrition.

#### Materials and methods.

The rabbits were housed in collective cages of 5 rabbits with a density of 15 rabbits/m\(^2\) in a closed unit in which the environment (temperature, lighting, and ventilation) was monitored and controlled. A total of 320 male and female rabbits were randomly assigned at weaning (age 35 d) to 1 of the 4 treatments. Thus, mean weights were similar between treatments and litter sizes were split to avoid any maternal effect. The HE100 and LE100 groups were then fed freely, and the LE75 and HE75 groups were given a restricted diet for 4 wk, until age 63 d. Afterwards, all 4 groups were fed freely until the end of the study at age 72 d. Moreover, 32 rabbits per group were immunized with ovalbumin as described below (*Figure 1*).

The amounts of feed distributed to the restricted groups 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–feed groups (LE100 and HE100) for periods of 3–4 d. Feed was given daily in a single distribution between 0800 and 0830, and water was consumed ad libitum. Rabbits were handled according to recommendations for the care of experimental animals in accordance with French national legislation (13).

Rabbits were weighed individually at weaning (age 35 d), after 1 wk of feed restriction (age 42 d), during the restriction period (age 50 d), at the end of the restriction period (age 63 d), and at the end of the study, after 9 d of ad libitum feed consumption by all 4 groups (age 72 d). The health status of the rabbits was assessed by monitoring for clinical signs of digestive disorders, such as diarrhea, cecal impaction, and suspicion of epizootic rabbit enteropathy, or other pathologies such as respiratory problems and injuries. Rabbits without clinical signs of illness but showing weight loss or very low growth (3 SD below the mean) were considered morbid and excluded from the study.

#### Ovalbumin immunization.

At age 37 d, 32 rabbits per group were immunized with a 1 mL subcutaneous injection of 600 μg ovalbumin (Sigma-Aldrich) in incomplete Freund’s adjuvant (Sigma) and challenged 9 d later (age 46 d) with 300 μg ovalbumin in incomplete Freund’s adjuvant. The weights (means and SDs) at age 33 d of the rabbits selected for immunization and those of the remaining rabbits were equivalent in order to have a representative sample of the study rabbits.

#### Killings and samplings.

Ten healthy rabbits per treatment were killed at ages 42, 50, 63, and 72 d. All rabbits killed at ages 63 and 72 d had been immunized with ovalbumin, whereas those killed at ages 42 and 50 d had not been immunized. The weights (means and SDs) of the rabbits selected for sampling and those of the remaining rabbits were equivalent in order to have a representative sample of the study rabbits. Rabbits were killed by electrical stunning followed by exsanguination. Blood was collected from the aorta in heparinized tubes (Vacuette 9 mL sodium heparin; Greiner Bio-One) and immediately stored on ice. After centrifugation (1000 \( \times \) g for 10 min at 4°C), plasma samples were stored at −20°C until further analysis. Lymphoid organs (spleen and vermiform appendix) were collected and weighed individually. Feces were collected from the rectums of the rabbits killed and transferred to dry tubes on ice until arrival at the laboratory, where the samples were stored at −20°C until further analysis. A 5 cm-long section of the ileum was collected from the ileocecal junction, snap frozen in liquid nitrogen, and stored at −80°C until analysis. The first day’s patch encountered from the ileocecal junction was isolated, the ileum was cut up, and a picture was taken of the patch next to a ruler. Images were analyzed through the use of ImageJ software (14) to evaluate the surface of the patches.

#### Total and anti-ovalbumin IgG ELISA measurements.

Concentrations of plasma immunoglobulin G (IgG) were measured by ELISA as described previously (15). Goat anti-rabbit IgG (Fc fragment–specific) was used as a capture antibody (Bethyl Laboratories) and HRP-labeled goat anti-rabbit IgG was used as a detection antibody in conjuction with a tetramethylbenzidine substrate (1:1 H\(_{2}\)O\(_2\):tetramethylbenzidine) (Thermo Fisher Scientific). The OD of each well was read at 540 nm and subtracted from the readings at 450 nm (Infinite M200, Tecan) to correct for 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. IgG concentrations were then obtained from the standard curve constructed with the OD values of an IgG standard solution.

Concentrations of specific anti-ovalbumin IgG were also measured by ELISA (16). Briefly, ELISA plates were coated with ovalbumin diluted

\textsuperscript{15} Abbreviations used: B2m, B2 microglobulin; DE, digestible energy; HE, high energy; HE100, high-energy ad libitum feed; HE75, high-energy restricted-feed; Hpt1, hypoxanthine phosphoribosyltransferase 1; LE, low energy; LE100, low-energy ad libitum feed; LE75, low-energy restricted-feed.

**Figure 1** Study design. AL, ad libitum feeding; BW, body weight; FR, feed restriction; HE100, high-energy ad libitum–feed group; HE75, high-energy restricted-feed group; LE100, low-energy ad libitum–feed group; LE75, low-energy restricted-feed group; OVA, ovalbumin immunization.
in carbonate buffer (4 g/L NaHCO₃, 0.1 mol/L pH 9.6) and incubated overnight at 4°C. Diluted plasma samples were then added to the plates, and the anti-ovalbumin antibodies were detected with HRP-labeled anti-rabbit IgG (Bethyl Laboratories). Plasma samples were quantified by reference to standard curves constructed with hyperimmune rabbit serum.

**Fecal IgA extracts and IgA ELISA measurements.** A small amount of fecal matter (200–600 mg) was diluted to 50 g/L in cold PBS. The samples were dispersed for 10 s on ice (T25 Ultra-Turax, IKA Labortechnik) and centrifuged at 3000 × g for 10 min at 4°C. The supernatants were then collected and stored at −20°C until analysis. Concentrations of plasma and fecal IgA were quantified by ELISA through the use of a specific polyclonal goat anti-rabbit IgA antibody (Bethyl Laboratories). Sample relative IgA concentrations were obtained from the standard curve constructed with the OD values of a diluted reference serum (Bethyl Laboratories).

**Expression of mRNA encoding for cytokines by real-time PCR.** Tissue RNA of 50- and 63-d-old rabbits was processed as previously described (17). Concentrations, integrity, and quality of RNA were determined spectrophotometrically (OD 260) through the use of the Nanodrop ND1000 (Labtech International). The sequences of primers used in the PCR for Il1b, Il2, Il8, Il10 and Tnfα are detailed in **Supplemental Table 2** and were purchased from Sigma. Real-time qPCR was performed in 384 well plates with the use of SYBR Green as the reporter. qRT-PCR data were expressed as 2^{-ΔΔCt} and normalized to a housekeeping gene. B2 microglobulin (B2m) was chosen among 3 other candidate reference genes [Gapdh, hypoxanthine phosphoribosyltransferase 1 (Hprt1), and cyclophilin A (Ppia)] because its expression was not affected by our experimental treatments or age, and it was thus considered to be a valid reference. Amplification efficiency and initial fluorescence were determined by using the same method as for real-time PCR (18). Finally, gene expression was expressed relative to the LE100 group. The specificity of qRT-PCR products was assessed at the end of the reactions by analyzing dissociation curves.

**Calculations and statistical analysis.** The DE contents of the diets were obtained from digestibility measurements presented in a companion paper (19). Mean DE intakes were then estimated for each treatment at each age as the mean gross intake per rabbit in the sampled cages, from weaning to killing, multiplied by the DE content of the diet for each treatment. Mean fecal IgA concentrations were plotted according to the OD values of a diluted reference serum (Bethyl Laboratories). Plasma samples were quantified by reference to standard curves constructed with hyperimmune rabbit serum.

Results

**Body weight and Peyer's patch surface area was lower with quantitative feed restriction without affecting relative spleen and appendix weight.** The feed intake of the rabbits consuming feed ad libitum averaged 135 g/d from age 35 to 63 d. The feed intake of the rabbits consuming the restricted diets was consistent with the level of 75% initially planned (75% and 74% for the LE and the HE diets, respectively, from age 35 to 63 d). As expected, growth rate was lower with feed restriction, leading to lower weight in the rabbits for which feed was restricted starting at age 42 d, whereas diet energy concentration did not affect growth (Figure 2).

The relative weights of each organ were not affected by the experimental treatments during the feed restriction period. However, the relative weight of the appendix was greater in rabbits fed the LE diet after 1 wk of ad libitum feed consumption at age 72 d (P < 0.05, 0.32% ± 0.01% compared with 0.28% ± 0.01%, data not shown). The surface area of the Peyer’s patches was not affected by the experimental treatments at age 42 and 50 d but was lower with feed restriction at age 63 d (P < 0.01, −0.2 cm²). After 1 wk of ad libitum feed consumption, the surface area of the Peyer’s patches of the rabbits for which feed previously was restricted was smaller than that of rabbits that consumed feed ad libitum throughout the study (P < 0.05, −0.2 cm²) (Figure 3). However, when the body weight of the rabbits was added as a covariable in our model, no effect from our treatments was detectable, regardless of age.

In conclusion, body weight was lower with feed restriction, but the growth of secondary lymphoid organs (spleen and appendix) and the surface area of the Peyer’s patches were not affected by feeding level, whereas dietary energy concentration only had a moderate effect on the growth of the appendix.

**Total and anti-ovalbumin plasma IgG concentrations were lower with quantitative feed restriction after 4 wk of treatment.** The main objective of this study was to assess the effects of feed intake level and dietary energy concentration on the immune response. Therefore, the systemic immune response, assessed by plasma IgG concentrations, was studied. Plasma total IgG concentrations were not affected during feed restriction (Figure 4). However, after returning to ad libitum feed consumption, rabbits that previously were restricted had lower IgG concentrations than rabbits that previously consumed feed ad libitum (−22%, P < 0.01). Concerning the effect of dietary energy concentration, after 1 wk of the study, IgG concentrations were higher in rabbits fed the HE diet than in those fed the LE diet (+23%, P < 0.05), but at all subsequent time points (50, 63, and 72 d), the diet energy concentration did not affect plasma IgG concentrations (Figure 4).

The ovalbumin immunization protocol allowed us to investigate the effects of feed intake level and dietary energy concentration on

![FIGURE 2](https://academic.oup.com/jn/article-abstract/145/3/483/4743688)
antigen-specific immunity. Plasma anti-ovalbumin IgG concentrations were lower with feed restriction at age 63 d (<41%, *P* < 0.05) (Figure 5), but after 1 wk of ad libitum feed consumption, previous feeding levels did not affect specific antibody concentrations. Diet energy concentration did not affect anti-ovalbumin IgG concentrations in any of the age groups. Taken together, these results demonstrate that feed restriction and dietary energy concentration had a limited effect on plasma total IgG concentrations, but feed restriction greatly affected treatment-specific anti-ovalbumin IgG production after 4 wk of treatment.

**Quantitative feed restriction reduced fecal and plasma IgA concentrations to a greater extent than did energy restriction.** Another aim of this study was to assess the effect of feed intake level and dietary energy concentration on the local immune response in the digestive tract through measurements of fecal IgA concentrations. Fecal IgA concentrations were lower during feed restriction (−40% at age 42 d, *P* < 0.05; −52% at age 50 d, *P* < 0.001; and −65% at age 63 d, *P* < 0.001) (Figure 6A). When rabbits returned to ad libitum feed consumption, no effect from the previous feeding levels was observed. Fecal IgA concentrations were higher in rabbits fed the HE diet than in those fed the LE diet from age 50 d onward (+56% at age 50 d, *P* < 0.01; +46% at age 63 d, *P* < 0.05; and +73% at age 72 d, *P* < 0.05), whereas the relative weight of the spleen decreased with age (−15%, *P* < 0.01) for all groups, whereas those of TNF-α were lower with feed restriction (−15%, 0.05 ≤ *P* < 0.1). Feed restriction did not affect the expression of IL-8 and IL-10 (Table 1).

**Immunologic values were affected by age.** The relative weight of the spleen decreased with age (*P* < 0.01) for all groups, whereas the relative weight of the appendix increased with age until age 63 d and decreased afterwards for all groups except LE75, for which age did not affect the relative weight of the appendix (Supplemental Table 3). The surface area of the Peyer’s patches moderately increased with age for all groups (*P* < 0.01) (Supplemental Table 3). Fecal IgA concentrations increased with age (*P* < 0.01) for the HE100 and HE75 groups but were unaffected by age for the LE100 and LE75 groups (Supplemental Figure 1A). Plasma total IgG and IgA concentrations increased with age regardless of study group (*P* < 0.001) (Supplemental Figure 1B and C).

**Discussion**

Dietary strategies influence animal health. The present study aimed to analyze the effects of quantitative feed restriction and dietary energy concentration on the immune response of
We demonstrated that feed restriction rather than dietary energy concentration is responsible.

The spleen, the appendix, and Peyer’s patches are major secondary lymphoid organs. As previously demonstrated in mice (20), feed restriction did not affect the relative weight of the spleen or the appendix. Dietary energy concentration had only a very moderate effect on the relative weight of the appendix at the end of this study. Similarly, the smaller surface area of the Peyer’s patches in the rabbits consuming a restricted diet may be correlated to the lower weight of these rabbits. Feed restriction and energy restriction therefore did not impair growth of the secondary lymphoid organs. Similar results were reported by Rogers et al. (20), who showed that a 30% reduction in feed intake did not affect the number of Peyer’s patches or their number of cells. However, Kubo et al. (21) demonstrated that 50% feed restriction reduced the number of cells in the spleens of growing mice. Thus, immune activity might be compromised by feed restriction despite the lack of effect on the physiologic variables of the secondary lymphoid organs measured.

Concerning the adaptive systemic immune response, feed restriction affected total IgG concentrations only after its application. A similar delayed dietary effect was reported by Zhu et al. (10) in growing rabbits fed diets differing in starch and fiber concentrations. As for the effects of feed restriction on the immune response to a specific antigen, the response was much more prompt: specific anti-ovalbumin concentrations were considerably lower after 4 wk of feed restriction. Similar results were obtained by Martin et al. (22) in 30% feed–restricted deer mice submitted to a keyhole limpet hemocyanin challenge. These findings could indicate that immunologic memory is an energetically costly process, which is negatively affected by feed restriction. Another hypothesis could be that antibody production is indirectly lowered through variations in nutrient or metabolite signals caused by reduced feed intake. Surprisingly, previous feeding level had no effect on the specific response to ovalbumin after 1 wk of ad libitum feed consumption. This could indicate that the specific immune response is readjusted very quickly after a change in the amount consumed.

The present study evaluated the effect of feed intake level and dietary energy concentration on the local immune response through fecal and plasma IgA concentrations and ileal cytokine expressions. Fecal IgA concentrations were lower with feed restriction in growing rabbits, confirming the results obtained in intestinal fluids and submandibular gland cultures in mice subjected to long-term feed restriction (8, 23). However, to our knowledge, this is the first study to find effects of short-term feed restriction on fecal IgA concentrations. As we published previously (19), DE intake was greater with the HE diet and ranked in the following order: LE75 < HE75 < LE100 < HE100. In this study a linear relation between DE intake and fecal IgA concentrations was found. We could therefore hypothesize that fecal IgA concentration might be regulated by DE intake rather than the quantity of feed ingested. However, the regression analyses were based on the mean energy intake of a sample of rabbits and not on individual measurements. Thus, additional studies would be necessary to confirm our hypothesis. Muthukumar et al. (23) showed that a 40% reduction in feed intake resulted in lower submandibular gland IgA concentrations and attenuated the onset of diseases in autoimmune-prone mice, indicating a possible protective effect from a reduction in IgA concentrations. The mycotoxin deoxynivalenol also induces an increase in IgA levels in mice (24) and is associated with intestinal lesions in pigs (25). Thus, the lower fecal IgA concentrations could be related to the lower incidence of digestive disorders observed in rabbits consuming the restricted diet and detailed in our companion paper (19). In addition, our results showed that plasma IgA concentrations were lower with feed restriction after 4 wk of treatment. The change in plasma IgA concentrations lagged behind the one occurring at the fecal level (age 63 compared with 42 d), suggesting that the immune response in peripheral blood occurs subsequent to the response in the digestive tract. This hypothesis is consistent with the results obtained in plasma IgG concentrations in our study and the study by Zhu et al. (10).
TABLE 1  Effect of feed intake level and dietary DE concentration on cytokine relative expressions in the ilea of growing rabbits between ages 50 and 63 d

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<th>LE75</th>
<th>HE100</th>
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1 Values are means and pooled SEMs, n = 10. DE, digestible energy; HE100, high-energy ad libitum-feed group; HE75, high-energy restricted-feed group; LE100, low-energy ad libitum-feed group; LE75, low-energy restricted-feed group.
2 All interactions between factors were nonsignificant.

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