# EFFECT OF DIETARY LIGNIN/STARCH RATIO ON THE NUMBER OF M CELL IN APPENDIX OF GROWING RABBITS

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## ABSTRACT

This study aimed to analyse the effect of dietary lignin /starch ratio on the number of M cell in appendix of growing rabbits. Two hundred weanling rabbits of both sexes (half/treatment), of 35 d old, were blocked by fifty healthy rabbits per group after a 7-d adaptation period and assigned to the 4 experimental diets by average live weight. Four different lignin to starch ratio diets I (0.34), II (0.28), III (0.22), IV (0.14) were formulated. Five rabbits per group were killed to require samples, every 10 days from52 to 82 days old. Vimentin immunoreactivity was determined the M cell number in the follicle-associated epithelium (FAE) of appendix. Significant interactions between age and diets were found. Rabbits fed the high lignin / low-starch diet (diet I) had a higher number of M cell in appendix than other diets at 52 days of age ($P$<0.001). A similar effect was observed at 62 days of age. In contrast, when dietary lignin/starch ratio decreased to 0.14, a significant decrease of the M cell number in appendix was observed at 52 days of age ($P$<0.001). With age, except diet I, the M cell number were similar between other diets ($P$>0.05) at 62 days of age. At 72 and 82 days of age, there was no difference in M cell number among four diets (P>0.05). Thus, a high lignin/low-starch diet would induce a M cell number increase at early stage of the experiment. With age, the intestinal environment would gradually adapt to the dietary components change, and the local mucous immunity seemed not affected by diet.

**Key words**: Rabbit, Dietary lignin/starch ratio, M cells, sacculus rotundus, Appendix.

## INTRODUCTION

The rabbit is a monogastric herbivorous animal, dietary fibers are the main constituents of its' feed, since it is adapted to a high intake of plant cell walls. It has been demonstrated that a high level of fiber leads to a decreased incidence of digestive troubles (Gidenne *et al.*, 2010). The symptoms sometime revealed injured intestinal mucous membrane where are distributed a plenty of lymphoid tissue (GALT). GALT is composed of solitary and aggregated lymphoid structure (Newberry, 2008). It is involved in the development of an immune response or tolerance to the antigens and pathogens found in the intestinal tract. The GALT of the rabbit include Peyer's patches, the *sacculus rotundus* (SR), appendix and caecal patches (Gebert and Bartels, 1991; Haley, 2003). The GALT is lined by follicle-associated epithelium (FAE) (Shaykhiev and Bals, 2007) which primary consists of membranous epithelial (M) cells and enterocytes. These tissues are antigen-sampling and inductive sites of the mucosal immune system. At these sites, the M cells transport antigens across the mucosal epithelial barrier to prime underlying lymphocytes for a subsequent immunological response (Didierlarurent *et al.*, 2002). Compared to other mammalian species, M cells of rabbits' Peyer's patches represent 50% of the FAE cell population. M cells are characterized by a basolateral cytoplasmic invagination that creates a pocket containing lymphocytes and occasional macrophages. The M cells are identified by vimentin expression. Vimentin, which is an intermediate filament, is generally found in cells of mesenchymal origin (Feyzullah *et al.*, 2010). Vimentin immunoreactivity was determined in both the perinuclear cytoplasm and cytoplasmic parts surrounding the intraepithelial lymphocytes (IEL) (Feyzullah *et al.*, 2010). Microorganism infection can result in an increase in both M cell size and in the proportion of the FAE surface area occupied by this cell type, which supported by the bulk of experimental evidence (Clark and Jepson, 2001a; Sansonetti *et al.*, 1996; Borghesi *et al.*, 1999; Meynell *et al.*, 1999). The gastrointestinal duct is exposed to numerous antigens and pathogenic agents throughout the life period of the organism (Brandtzageg *et al.*, 2008).

So, the M cell changes would reflect on the body's immunity status, for example in response to different diets.

By an analysis of the vimentin expression in the FAE of the appendix, we aimed to study the effects of age and increasing the dietary lignin/starch ratio on the number of M cell in appendix of growing rabbits, to understand the effects of diet on mucous immunity.

## MATERIALS AND METHODS

### Animals and experimental design

Two hundred weanling rabbits of both sexes (half/treatment) of 35 d old, weighing 1030 ± 55g of BW, were equally assigned at 42d old, after a 7-d adaptation period, to the 4 experimental diets (50 rab. per diet) and allotted according to live weight at 42d old. Four different lignin to starch ratio diets I (0.34), II (0.28), III (0.22), IV (0.14) (Table1) were formulated and pelleted. Rabbits were caged per two (size of cage: 40×40×50cm) and had *ad libitum* access to the feed and water. They were housed in an environmentally semi-controlled closed building during the experimental period. Temperature conditions were maintained between 15 and 25°C.

Five rabbits per group were killed to require appendix every 10 days from 52 to 82d old. After paraformaldehyde fixation, the tissues were embedded in paraffin. The 5 µm-thick sections cut from the paraffin blocks were obtained to observe of the general structure. Sections were placed on poly-L-lysine slides, and then carried on immunohistochemical experiment according to the instruction of Plink-2 plus Polymer HRP Detection System for Mouse Primary Antibody (PV-9002). Monoclonal mouse anti-vimentin (Clone V9, DAKO) was primary antibodies and antigen-antibody reaction was made visible by using DAB, and later Gill's hematoxylin was applied for background staining. While all immunohistochemical process were performed according to the method reported by Feyzullah *et al.* (2010).

### Chemical analyses

Diets were analyzed following the recommendation of the Association of Official Analytical Chemists (National Standards Recommend Method, China). Starch concentration was measured by polarimetry. Crude fibre was determined using Acid-base method. NDF and ADF use detergent method of Van-Soest.

**Table 1:** Composition of the experimental diets[a]

| Items | Diet I (%) | Diet II (%) | Diet III (%) | Diet IV (%) |
|---|---|---|---|---|
| Dry matter | 87.7 | 87.8 | 87.5 | 87.5 |
| Neutral detergent fibre (NDF) | 27.2 | 27.3 | 26.7 | 21.0 |
| Acid detergent fibre (ADF) | 18.0 | 17.1 | 16.0 | 11.5 |
| Acid detergent lignin (ADL) | 4.9 | 4.6 | 4.2 | 3.6 |
| Starch | 14.5 | 16.4 | 19.2 | 25.0 |
| Ash | 9.6 | 7.8 | 8.8 | 10.1 |
| Crude protein | 17.4 | 17.4 | 18.4 | 18.3 |
| Calcium | 1.3 | 1.3 | 1.1 | 1.1 |
| Phosphorus | 0.7 | 0.7 | 0.7 | 0.7 |
| **ADL/Starch** | **0.34** | **0.28** | **0.22** | **0.14** |

[a] Dry matter basis

### Statistical analysis

Ten vimentin-positive sections selected from the rabbits' appendix of same diet and same age, and three fields of view selected from same section were analyzed by ImagJ (National Institutes of Health, USA). This software was used to determine the grey level among immuno-reactivity observed in the samples. The

grey level has a negative correlation with positive cell number (M cell). Statistical analysis of data was performed by ANOVA for a completely randomized block design with same treatment as block and the diet (ADL/starch) and treatment time (age) as the two main source of variation by using the generalized linear model procedure of SAS (SAS Inst. Inc., Cary, NC, USA).

The liner model formula is $X_{ijk}=\mu+\alpha_i+\beta_i+\gamma_{ij}+e_{ijk}$, $X_{ijk}$ represent the M cell mean grey level; $\alpha_i$ represent the different diet treatment; $\beta_i$ represent the different feeding days; $\gamma_{ij}$ representing diets × age. Significance was declared at $p \leq 0.05$. Since significant interactions were detected among diet and age, mono-factorial variance analysis were done, within each age, to compare the means from the four diets.

## RESULTS AND DISCUSSION

The M cell number of the appendix was affected by diet ($p$<0.001). In particular, the rabbits' appendix in diet I had a higher number of M cell compared with other diets ($P$<0.001, Table 2). According to the grey level in negative correlation with positive cell (M cell), so the M cell number of diet I is the largest among the different diets. For age, the number of M cell in the appendix was up to maximum at 72 days of age ($P$<0.001). Highly significant interactions were detected among diets and age for the expression of M cells in appendix (P<0.001; Table 2). The highest lignin/starch ratio (diet I, 0.34) had higher the number of M cell in appendix than other diets at 52days of age ($P$<0.001, Table 3) . A similar effect has been observed at 62 days of age. In contrast, when dietary lignin/starch ratio decreased to 0.14 (diet IV) in rabbit diet, a significant decrease of the M cell number in appendix was observed at 52 days of age ($P$<0.001). Except diet I, the M cell number affected by other diets did not show difference ($P$>0.05) at 62 days of age. With age, the difference was not detectable among four diets (P>0.05) from 72d to 82 days of age. In the present study, we found that high lignin can induce M cell number increasing in the early stage of the experiment to improve the intestinal mucous immunity.

**Table 2**: Effect of diets and age on M cell numbers in rabbits appendix *****

| Diet | | | | Age (days) | | | | P level | | |
|---|---|---|---|---|---|---|---|---|---|---|
| I | II | II | IV | 52 | 62 | 72 | 82 | Age | Diet | A x D |
| 119.3c ±0.8 | 125.8b ±1.1 | 125.7b ±1.1 | 129.7a ±1.2 | 129.1A ±1.8 | 124.9B ±0.8 | 121.9C ±0.7 | 124.5B ±0.7 | < 0.001 | <0.001 | < 0.001 |

n= 30 views in immunohistochemical sections all treatment rabbits per group; Means ±SEM
[a-d] Mean values with a different letter, and within diet or age, differ at the level P<0.05

**Table 3** Effect of diets × age interaction on the M cell numbers in appendix of the rabbits*****

| Diets | 52d | 62d | 72d | 82d |
|---|---|---|---|---|
| I | 109.66[a]±1.66 | 118.97[a]±1.12 | 123.98±1.2 | 124.0±1.0 |
| II | 133.9[e]±3.11 | 126.33[bc]±1.48 | 120.68±1.24 | 122.74±1.7 |
| III | 129.17[bc]±3.24 | 126.59[c] ±1.53 | 122.33 ±1.71 | 125.13 ±1.23 |
| IV | 145.03[d]±2.35 | 127.58[dc] ±1.49 | 120.44 ±1.65 | 126.38 ±1.33 |

n= 30 views in immunohistochemical sections all treatment rabbits per group; Means ± SEM
[a,b,] Mean values within a column without a common superscript letter differ at the level P<0.05.

Another study in rabbits also reported that a dietary *"soluble"* fiber may have a protective effect upon the mucosa and would favor the immune response (Gómez-Conde *et al.*, 2007). However, wwith age, the intestinal environment seemed to adapt gradually to the dietary components change. Therefore, local mucosal immunity status might tend to be stable.

## CONCLUSIONS

According to our results, the highest ADL/starch ratio (0.34) had the lowest number of M cell but only in the early stage of rabbits' growth. With age, the different dietary lignin/starch ratio did not affect M cell number in appendix of growing rabbits.

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## REFERENCES

Brandtzaeg P., Kiyono V., Pabst R., Russsel M.W. 2008. Terminology: nomenclature of mucosa-associated lymphoid tissue. *Mucosal Immunol. 1, 31-37.*

Didierlaurent A., Sirard J.C., Kraehenbuhl J.P., Neutra M.R. 2002. How the gut senses its content. *Cell Microbiol. 4, 61-72.*

Feyzullah B., Ergün E., Bayraktaroğlu A.G. 2010. The identification of intestinal M cells in the sacculus rotundus and appendix of the Angora rabbit. *Vet Res Commn. 34, 255-265.*

Gidenne T. 2003. Fibres in rabbit feeding for digestive troubles prevention: Respective role of low-digested and digestible fibre. *Livest. Prod. Sci., 81, 105-117.*

Gidenne T., García J., Lebas F., Licois D. 2010. Nutrition and Feeding Strategy:Interactions with Pathology. *In: De Blas C., Wiseman J. (Eds). The Nutrition of the Rabbit. CABI Publishing. CAB International, Wallingford Oxon, UK, 179-199.*

Gómez-Conde M.S., Garcia J., Chamorro S., Eiras P., Rebollar P., Pérez de Rozas A., Badiola I., De Blas J.C., Carabaño R. 2007. Neutral detergent-soluble dietary fiber improves gut barrier function in 25 day old weaned rabbits. *J. Anim. Sci. 85, 3313-3321.*

Newberry R.D. 2008. Intestinal lymphoid tissues: is variety an asset or a liability. *Curr Opin Gastroenterol. 2, 121-128.*

Shaykhiev R., Bals R. 2007. Interactions between epithelial cells and leukocytes in immunity and tissue homeostasis. *J. Leukoc. Biol. 1-15.*