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## Effect of Citric Acid Supplementation on Nutrients Digestibility, Nutritive Values and Intestinal Histomorphology of Growing Rabbits

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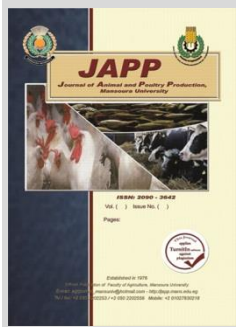
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### ABSTRACT

Fifteen of New Zealand White rabbits that are 12 weeks old, weighing  $2064 \pm 31.78$  g were used in this study. Rabbits were divided into five similar groups and housed in separate cages. The 1st pelleted experimental diet (R1) was used as a control; the supplemented citric acid was (0.5, 1.0, 1.5 and 2.0% of the R1 diet for R2, R3, R4 and R5 diets, respectively). The nitrogen-free extract digestibility values were higher ( $p < 0.05$ ) by feeding on R2 or R3 than feeding on R5 diet (83.21, 82.08 and 70.52%, respectively). However, the feeding on R1 or R2 showed the highest ( $p < 0.05$ ) ratio of digestible energy intake to digestible crude protein intake (DEI/DCPI) (20.82 and 20.68, respectively). The villus height values were higher ( $P < 0.05$ ) with feeding on R2, R3, R4 and R5 than feeding on R1 diet. Submucosa thickness was decreased ( $P < 0.05$ ) with feeding R2 as opposed to feeding on the other diets. The feeding on R4 showed that there are some few aggregations of inflammatory cells in between the crypt, while feeding on R5 showing multifocal, mild inflammatory aggregations around single enterocyte damage. In conclusion, the current results showed that feeding on R1 or R2 were better for DEI/DCPI, villus height and the sub mucosa thickness. However, there is little knowledge on how citric acid affects rabbits, therefore further research is needed.

**Keywords:** digestibility, nutritive values, citric acid and growing rabbit.



### INTRODUCTION

In the presence of an alkaline gut environment in rabbits, digestive enzymes cannot function properly, resulting in reduced nutrient digestion and absorption (Schnabel *et al.*, 1982). Therefore, it is important to find alternative feed additives that can promote growth, enhance digestibility, inhibit microbial growth, and have no negative effects on rabbit health or human consumption of their meat. Adding organic acids to the rabbit diet could be beneficial because weaned rabbits have limited capacity to acidify their stomach contents, leading to suboptimal protein digestion and inadequate pathogen elimination due to low pH levels (Johnson-Delaney, 1996).

Incorporating organic acids into the diet reduces the feeds' ability to act as a buffer, enabling the stomach contents to achieve an ideal pH of about 3 more quickly. This pH level is essential for the efficient activity of the proteolytic enzyme pepsin (Blanchard and Wright, 2004; Davidson *et al.*, 2001). The dissociated portion of organic acids can easily penetrate the cell wall of microbial cells, particularly gram-negative bacteria, and dissociate inside the cells, lowering the pH and causing detrimental effects on the bacteria. The bacteria's attempts to eliminate the released protons ( $H^+$ ) would deplete their metabolism, and the remaining anion could damage the cytoplasm or cell nucleus, resulting in the death of the bacteria (Dam, 2006). Certain organic acids exhibit stronger antimicrobial properties than others, with medium-chain fatty acids being particularly effective (Decuypere and Dierick, 2003).

Organic acids help maintain the integrity of the gut lining and improve digestion by promoting a healthy gut flora (Sultan *et al.*, 2015). The cost of feed per bird was increased when 1.0% of citric acid was introduced, while the lowest cost was for the control group with no citric acid, according to Sharifuzzaman *et al.* (2020).

Citric acid has been shown to stimulate the growth of intestinal mucosa, improving nutrient digestion and animal performance (Romero *et al.*, 2016). Previous studies (Pelicano *et al.*, 2005) have reported that the villas were higher in the intestinal when using organic acidifiers. This is explained by the fact that both pathogenic and non-pathogenic gut bacteria grow less rapidly when exposed to organic acids. Reduced colonization and infectious processes ultimately result in a decrease in inflammatory responses in the intestinal mucosa, which also enhances mucosal nutritional absorption.

There are limited experiments have been done on feeding organic acids to rabbits, with mixed outcomes thus far. However, rabbits fed a diet containing 0.4% formaxol-encapsulated formic acid, citric acid, and essential oils in micro-granule form showed no detection of pathogenic bacteria, which was attributed to the low pH in the cecum (Emmy, 2008).

In order to better understand the apparent nutrient digestibility and intestinal histomorphology of growing New Zealand White rabbits fed diets supplemented with various doses of citric acid, this study looked at both of those factors.

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## MATERIALS AND METHODS

The research was conducted at the Experimental Station in El-Serw, Agriculture Research Center, Ministry of Agriculture, Egypt. The aim of this study was to examine the impact of different amounts of citric acid in diets on the digestibility, and intestinal histology of New Zealand White rabbits.

### Experimental animals and management:

Fifteen of White New Zealand rabbits used in the research that were 12 weeks old. They were each kept in steel cages that were 50 x 50 x 45 cm in size and provided with fresh water through automatic nipple drinkers and manual feeders. The rabbits had unrestricted access to feed and water. The rabbits were randomly divided into five groups, each consisting of three rabbits. The basic diet's ingredients complied with the recommendations of De Blas and Mateos (2020) as shown in Table 1. The chemical composition of the basal diet without citric acid was determined following the standards of the Association of Official Analytical Chemists (AOAC, 2006).

The following five experimental meals for pellets were used: R1 = the experimental basal diet; R2 = R1 + 0.5% citric acid; R3 = R1 + 1.0% citric acid; R4 = R1 + 1.5% citric acid; and R5 = R1 + 2.0% citric acid.

**Table 1. Composition and chemical analysis of the experimental basal diet.**

Ingredient	Basal (%) diet
Alfalfa hay	31.0
Barley grain	24.6
Wheat bran	28.0
Soybean meal	13.25
Molasses	2
Limestone	0.95
Dicalcium phosphate	1.6
Sodium chloride	0.3
Vit. Min. Premix*	0.3
Total	102
DM (%)	86.04
Chemical analysis (as % on dry matter basis)	
OM (%)	92.02
CP (%)	19.96
CF (%)	14.18
Ether extract (%)	2.55
NFE (%)	55.33
DE (kcal/kg)	2411
Metabolizable energy (kcal/kg)	2215
Calcium (%)	1.20
Total phosphorus (%)	0.76
Lysine (%)	0.83
Methionine	0.24

\*One kilogram of minerals–vitamins premix provided as: Vitamin K 3.21 mg; Vitamin E, 100 mg; Vitamin A, 150,000 IU; Vitamin B2.40 mg; Vitamin B6, 15 mg; Vitamin B12, 0.1 mg; Vitamin B1, 10 mg; Niacin, 200 mg; Pantothenic acid, 100 mg; Biotin, 0.5 mg; Folic acid, 10 mg; Choline chloride, 5000 mg; Zn, 450 mg; Fe, 0.3 mg; Mn, 600 mg; Co, 2 mg; Cu, 50 mg; and Se, 1 mg.

### Nutrient digestibility:

To estimate the apparent nutrient digestibility coefficients, feed and fecal samples were collected. Fecal samples were collected daily for an additional week before the morning feeding and stored at -20°C. The samples were dried, ground, and chemically analyzed for dry matter (DM), crude protein (CP), ash, crude fiber (CF), ethereal extract (EE), and nitrogen-free extract (NFE) using AOAC (2019) methods. Digestion coefficients and feeding values were calculated for each experimental group based on the NRC (2001) guidelines.

Using AOAC (1990) techniques, a wide range of nutrients were examined in the diets and fecal samples. Both digestible crude protein (DCP) and total digestible nutrients (TDN) were calculated in accordance with to McDonald *et al.* (1973), while the digestible energy (DE) was determined using the formula: DE (kcal/kg DM) = [(5.28 × DCP) + (9.51 × DEE) + (4.20 × DCF) + (4.20 × DNFE)] (Schiemann *et al.*, 1972).

### Intestinal morphology:

At 13 weeks of age, the rabbits were humanely euthanized, and their small intestines were carefully extracted. The intestines were cut into 2 cm-long segments, cleaned, and left to dry for 24 hours in a 10% formalin solution. Samples were produced, stained, and used, light microscope (Olympus, Tokyo, Japan) and stained with hematoxylin and eosin (Bancroft *et al.*, 2012).

### Statistical analysis:

The SAS (2000) General Linear Model Program was used to evaluate the data gathered, and Duncan's Multiple Range Test (Duncan, 1955) was applied to find differences between the means of the treatments. The productive traits of the rabbit groups underwent oneway analysis of variance using the mathematical model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

Where  $Y_{ij}$  represents the observed factor,  $\mu$  is the overall mean,  $T_i$  is the effect of citric acid level, and  $e_{ij}$  is the random experimental error.

## RESULTS AND DISCUSSION

Table 1 presents the composition and calculated analysis of the basal diet used in the experiment. Hillyer *et al.* (1997) emphasized the importance of pelleted rabbit diets, they are made up of several substances that have been combined to offer the necessary nutrients. Alfalfa hay, cereals and grain byproducts, protein, mineral, and vitamin supplements are typical components of the basic diet.

The chemical nutrient levels in the basal diet were appropriate for the formulation of diets for growing rabbits, as found by Gidenne (1992) and De Blas *et al.* (1995). These recommendations suggested a dietary range of 16.3% to 19.8% crude protein (CP), 14% to 18% crude fiber (CF), and 5.5% ethereal extract (EE).

The feeding values and nutritional digestibility were significantly impacted by the experimental meals (Table 2). In growing rabbits, nitrogen-free extract (NFE) had a better level of digestibility ( $p < 0.05$ ) when fed diets R2 or R3 compared to the R5 diet (83.21%, 82.08%, and 70.52%, respectively). Regarding feeding values, an increase in citric acid level in the diet affected ( $p < 0.05$ ) the digestible crude protein. However, diets R1 and R2 showed the highest ( $p < 0.05$ ) DEI/DCPI ratio (20.82 and 20.68, respectively) compared to other groups (Figure 1). The digestibility of ethereal extract (EE) increased ( $p < 0.05$ ) with feeding on R2 (3.22%) compared to the control group (1.05%). There were no significant effects observed among the experimental diets on the digestibility of crude fiber (CF), NFE, or total digestible nutrients (TDN).

The caecum, which constitutes 40% of the rabbit's entire digestive tract, plays a vital role in digestion as a major site of fermentation and fiber degradation (pH 5.4 - 6.8) (Garcia *et al.*, 2002). The microbial activity in the caecum is crucial for rabbit digestion and overall health.

The main source of energy for rabbits is their maintenance requirements, which range from 2100 to 2200 Kcal/kg. Healthy rabbits typically consume enough food to

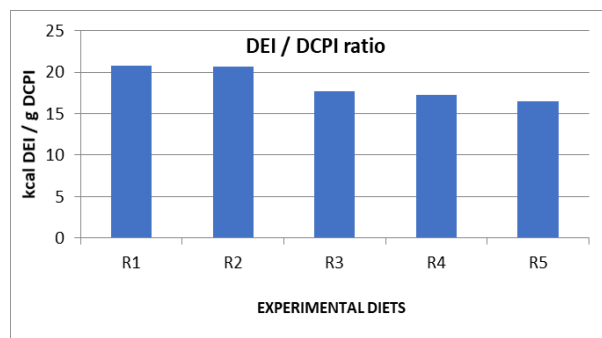
meet their digestible energy (DE) needs. Feed intake is higher when rabbits are on a low-energy diet, while it decreases with a high-energy diet. The amount of crude protein in the diet is

influenced by digestion capacity and DE content. To optimize growth rate and reduce mortality, it is recommended to maintain a ratio of [(23.5 Kcal DE/g DP) or (10 g DP/Mj DE)].

**Table 2. Effect of feeding experimental diets without or with citric acid on nutrient digestibility and feeding values.**

Items	Citric Acid					SEM
	R1 0.0%	R2 0.5%	R3 1.0%	R4 1.5%	R5 2.0%	
DM intake (g/h/d)	182.74	178.91	177.75	172.51	176.44	
Nutrient digestibility (%):						
DM	61.11	61.81	62.66	58.36	56.61	2.56
OM	63.76	64.82	65.71	61.24	59.23	2.40
CP	58.11	60.00	67.71	64.89	65.70	2.25
CF	39.88	36.57	31.03	32.80	30.62	6.07
EE	29.16	56.40	44.46	24.64	38.42	10.16
NFE	79.74 <sup>ab</sup>	83.21 <sup>a</sup>	82.08 <sup>a</sup>	75.19 <sup>ab</sup>	70.52 <sup>b</sup>	2.40
Digested nutrients and feeding value as DM (%):						
DCP	10.67 <sup>b</sup>	11.02 <sup>ab</sup>	12.73 <sup>a</sup>	12.07 <sup>ab</sup>	12.02 <sup>ab</sup>	0.41
DCF	6.09	5.58	4.62	5.14	4.65	0.92
DEE	1.05 <sup>b</sup>	3.22 <sup>a</sup>	1.67 <sup>ab</sup>	0.67 <sup>b</sup>	1.17 <sup>b</sup>	0.34
DNFE	45.22	46.53	46.70	42.79	40.51	1.37
TDN	63.04	66.36	65.77	60.67	58.36	2.13
DCPI (g/d)	19.55	19.79	22.61	20.84	21.40	1.93
TDNI (g/d)	115.44	119.64	117.02	104.94	103.63	10.99
DE kcal/kg	2221.71	2277.89	2239.49	2083.36	1971.48	81.62
DEI kcal/d	406.50	411.22	398.60	360.37	349.49	38.10
DEI/DCPI	20.82 <sup>a</sup>	20.68 <sup>a</sup>	17.64 <sup>ab</sup>	17.26 <sup>ab</sup>	16.48 <sup>b</sup>	0.84

a, b, c : Means within the same raw with different superscripts are significantly different (P < 0.05). SEM = standard error of means.



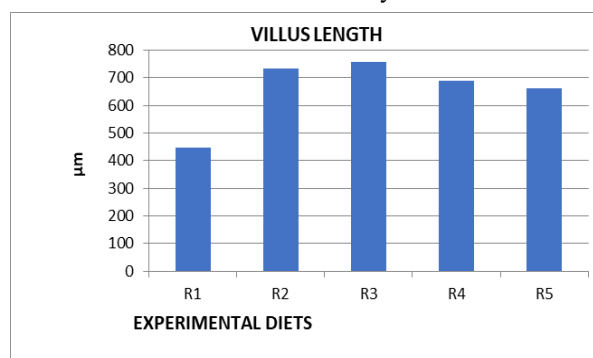
**Fig. 1. Effect of feeding experimental diets on growing rabbits**

Utilizing organic acids in the food lowers the feed's buffering ability, hastens the stomach's contents achieving the ideal pH level of around 3, and increases the pepsin enzyme's effectiveness (Blanchard and Wright, 2004; Davidson *et al.*, 2001). Organic acids also lower the pH of digesta, increase pancreatic secretion, and have positive effects on the intestinal mucosa (Dibner and Buttin, 2002).

Broiler chickens' small intestine villus height has been observed to increase with the use of organic acidifiers; this effect may be caused, in part, by a decrease in the growth of harmful and benign bacteria in the intestines of chickens (Pelicano *et al.*, 2005). As salivary amylase, which is inactive at pH 3.5, is responsible for carbohydrate breakdown in the stomach, pepsin functions best in an acidic environment with a pH of 2.0 to 3.5. The digestibility of ether extract (EE) was highest (p < 0.05) in

the 1.5% citric acid group and lowest in the 1.0% citric acid group. The control group showed the highest level of nitrogen-free extract (NFE) digestibility, while the lowest was in the 1.0% citric acid group. The addition of citric acid slightly increased the digestibility of crude protein (CP), crude fiber (CF), and ethereal extract (EE) in the diet (Uddin *et al.*, 2014).

The representative photomicrographs of the intestine from different treatment groups showed variations in villus height, villus width, submucosa thickness, and muscular thickness, as presented in table (3) and the figure (2) for villus length. Villus height values were higher (p < 0.05) in diets R2, R3, R4, and R5 compared to the R1 diet. There were some instances of edema separating the mucosal layers from the submucosa and muscular layers.



**Fig. 2. Effect of feeding experimental diets villus length**

**Table 3. Effect of feeding experimental rations without or with citric acid levels on intestinal histomorphology.**

Items	Citric Acid					SEM
	R1 0.0	R2 0.5%	R3 1.0%	R4 1.5%	R5 2.0%	
Villus height	445.88 <sup>b</sup>	731.14 <sup>a</sup>	758.26 <sup>a</sup>	689.90 <sup>a</sup>	659.60 <sup>a</sup>	42.6119
Villus width	106.80 <sup>bc</sup>	143.70 <sup>ab</sup>	92.74 <sup>c</sup>	114.50 <sup>bc</sup>	164.28 <sup>a</sup>	11.7667
Submucosa	51.04 <sup>a</sup>	29.51 <sup>b</sup>	49.27 <sup>a</sup>	42.20 <sup>ab</sup>	52.50 <sup>a</sup>	4.4143
Muscular	72.40 <sup>a</sup>	31.51 <sup>d</sup>	45.40 <sup>c</sup>	54.93 <sup>bc</sup>	58.98 <sup>b</sup>	2.4274

The villus width values showed that feeding on R3 or R4 were lowest (P < 0.05) than feeding on the other diets. Intestine villus normally arranged intestinal villi with normal goblet cell. Submucosa thickness was decreased (P

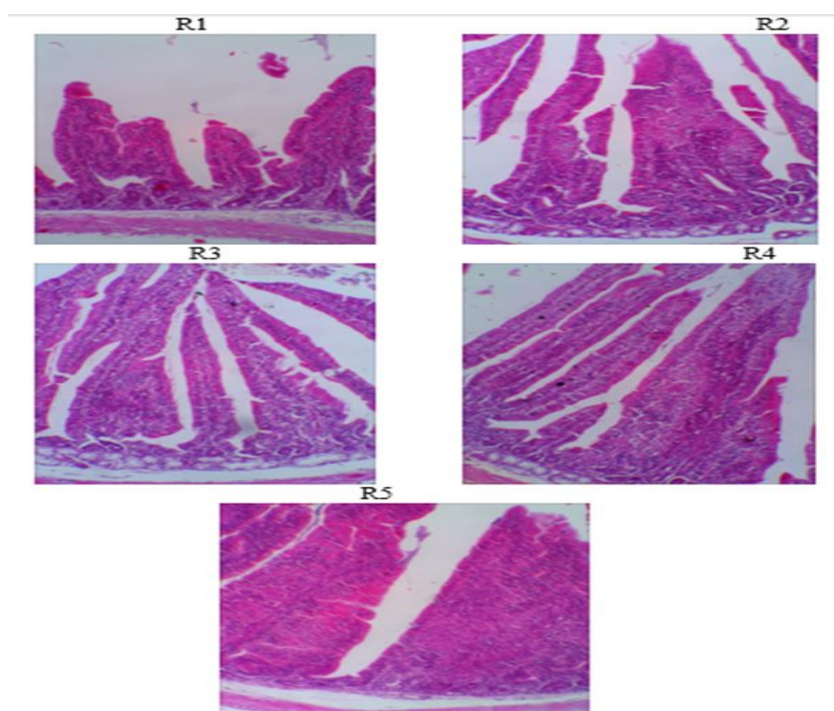
< 0.05) with feeding on R2 than feeding on the other diets. The feeding on R4 showed that there are some few aggregations of inflammatory cells in between the crypt, while feeding on R5 showing multifocal, mild inflammatory

aggregations around single enterocytes damage. Muscular thickness was higher ( $P < 0.05$ ) with feeding on R1 than feeding on R2, R3, R4 and R5 diets.

By encouraging a healthy balance of gut flora, organic acids help to preserve the integrity of the gut lining and enhance digestion (Sultan *et al.*, 2015). By adding 0.5% citric acid to the broilers' diet, Haque *et al.* (2010) found that weight gain, feed intake, tibia ash deposition, non-specific immunity, feed efficiency, and carcass yield all increased. Sharifuzzaman *et al.* (2020) found that the addition of 1.0% citric acid to the diet resulted in the highest feed cost per bird, while the control group with 0% citric acid had the lowest feed cost. Citric acid was shown to stimulate the growth of the intestinal mucosa, improving nutrient digestion and animal performance (Romero *et al.*, 2016).

These results are in line with those of an earlier study by Pelicano *et al.* (2005), which found that organic acidifiers raised villus heights in the duodenum and jejunum. The ability of organic acids to prevent the growth of both pathogenic and nonpathogenic bacteria in the intestines, hence lowering colonization and the occurrence of illnesses, can be linked to this rise in villus height. As a result, the intestinal mucosa's inflammatory responses are reduced, which improves the villus' height and the mucosa's abilities to secrete, digest, and absorb nutrients.

Afsharmanesh and Pourreza (2005), Shen-HuiFang *et al.* (2005), Jozefiak and Rutkowski (2005), and Boling *et al.* (2000) reported positive effect of citric acid on growth performance of broiler chicks and pig, but there is rare information on the effect in rabbits.



#### Representative photomicrograph of intestine from different treatment groups.

- R1: control group showing normally arranged intestinal villi with few edemas separated mucosal layers from sub mucosa. The muscular layers are with few aggregations of inflammatory cells in between the crypt, and shortest villus length.  
R2: showing longest intestine villus and normally arranged intestinal villi with normal goblet cell.  
R3: showing normally arranged intestinal villi with normal goblet cell and long intestine villus.  
R4: showing normally arranged intestinal villi with few aggregations of inflammatory cells in between the crypt.  
R5: the intestine group showing multifocal, and mild inflammatory aggregations around single enterocytes damage.

### CONCLUSION

The current findings address the fundamentals of citric acid, its mode of action, novel techniques to improve consumption, its impact on nutrient digestibility, and intestine histomorphology. The present results showed that feeding on R2 or R3 were better for digestible energy intake to digestible crude protein intake ratio, villus height and the sub mucosa thickness. However, there is scarce information on the effect of citric acid in rabbit which need some studies in the future.

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## تأثير إضافة حمض الستريك على معاملات الهضم والتركيب الهستولوجي للأمعاء الدقيقة في الأرانب النامية

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### الملخص

أجريت هذه التجربة على خمسة عشرة من الأرانب النيوزيلاندي البيضاء في عمر 12 أسبوعاً وبمتوسط أوزان  $31,78 \pm 2,64$  جم. وتم توزيع الأرانب إلى خمس مجموعات (ثلاثة أرانب في كل معاملة). وتم تكوين العليقة التجريبية الأولى (R1) كعليقة كنترول التي احتوت على: 31,0% دريس برسيم حجازي + 24,6% حبوب شعير + 28,0% نخالة قمح + 13,25% مسحوق فول الصويا + 2,0% مولاس + 0,95% حجر جيرى + 1,60% فوسفات ثنائي الكالسيوم + 0,30% كلوريد الصوديوم + 0,30% مخلوط معادن وفيتامينات وتم إضافة حامض الستريك في العلائق بمستويات (0,5، 1,0، 1,5، 2,0) % إلى العليقة التجريبية الأولى لتكوين العلائق التجريبية (R2، R3، R4، R5) على التوالي. وأهم النتائج المتحصل عليها كما يلي: زاد معامل هضم المستخلص الخالي من الازوت معنوياً عند التغذية على (R2) و (R3) مقارنة بالتغذية على العليقة (R5) (83,21 و 82,08 و 70,52 % على التوالي. بينما لم تظهر فروق معنوية بين R1 و R2 و R3 و R4. زاد البروتين الخام المهضوم بزيادة تركيز حامض الستريك. بينما كان معدل الاستفادة من الطاقة إلى البروتين المهضوم (DEI / DCPI) أعلى عند التغذية على (R1 و R2) (20,82 و 20,68 على التوالي) كما زاد المهضوم من مستخلص الاثير عند التغذية على R2 مقارنة بالتغذية على R1. زاد طول الخملات معنوياً بالتغذية على R2، R3، R4، R5 مقارنة R1 بينما انخفض عرض الخملات عند التغذية على R3، R4. انخفض معنوياً سمك الخملات بالتغذية على R2 مقارنة بالعلائق الأخرى. أظهرت التغذية على العلائق R4، R5 التهابات ببعض الخلايا. زاد سمك العضلات للأمعاء الدقيقة بالتغذية على العليقة R1 مقارنة بالعلائق الأخرى. أظهرت النتائج أن التغذية على R1 و R2 كانت الأفضل في الاستفادة من الطاقة المهضومة بالنسبة للبروتين المهضوم DEI / DCP وأدى إلى زيادة ارتفاع الخملات وسمك الغشاء المخاطي في الأمعاء الدقيقة ولكن مازال هناك الاحتياج إلى مزيد من دراسة تأثير حمض الستريك في الأرانب مستقبلاً.