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Thymol in fattening rabbit diet, its bioavailability and effects on intestinal morphology, microbiota from caecal content and immunity

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Abstract

To our knowledge, no study has been carried out to evaluate the effects of thymol sustained administration on gut health and immune response in terms of its bioavailability in the rabbit organism. A total of 48 rabbits were randomly divided at 35 days of age into two dietary treatment groups (C/control or T/thymol at 0.025% addition). Thymol was added for 21 days and then withdrawn for 7 days. Thymol content in faeces ($p < 0.001$) and caecal content ($p < 0.05$) was significantly higher than in plasma during the thymol addition and withdrawal (2442 ± 451.4, 881.9 ± 231.3 ng/g DM and 46.2 ± 28.4 ng/ml, respectively; 149.5 ± 40.54, 45.76 ± 12.44 ng/g DM and 2.73 ± 0.45 ng/ml, respectively). Increasing of villi height to crypt depth ratio in small intestinal wall ($p < 0.01$), phagocytic activity in blood ($p < 0.0001$) and lactic acid bacteria in caecal appendix ($p < 0.01$) and faeces ($p < 0.05$) was still presented after withdrawn of thymol. The thymol at this concentration demonstrated its biological properties and was able to positively affect gut health and immune response of rabbits.

KEYWORDS
bioavailability, immunity, intestinal morphology, microbiota, rabbits, thymol

1 | INTRODUCTION

It is well known that *Thymus vulgaris* is widely used as a medicinal plant particularly due to its special antimicrobial, antioxidant, antiparasitic and immunostimulatory functions. It has various beneficial effects on animal health, and much interest has been shown in its application in rabbit farms as a nutritional supplement to improve performance and meat quality. One of the major components of thyme essential oil is phenolic compound thymol (Abdel-Gabbar et al., 2019; Placha et al., 2019), which is mainly responsible for its beneficial properties.

In recent years a small number of studies has been published about thymol distribution in animal tissues. Mason et al. (2017) detected thymol in liver, kidney and fat of cattle, Zitterl-Eglseer et al. (2008) in kidneys of piglets, Haselmeyer et al. (2015) in plasma, muscle, liver and kidneys of broiler chickens; Oceľová et al. (2016, 2018) in intestinal wall and gut content of broiler chickens as well. To our knowledge though, no study has been carried out on thymol absorption and distribution in the rabbit organism.

Phytogenic feed additives are able to positively affect intestinal morphology in terms of villi height and villi/crypt ratio. One consequence of these histomorphological changes is more efficient absorption of nutrients. These studies need verification with bioactive substances derived from different plants (Bozkurt & Tüzün, 2020). Youssef et al. (2021) found that feeding chickens with plant essential

oils in a blend of star anise, rosemary, thyme and oregano efficiently improved their intestinal morphology by increasing the villi height and the villi/crypt ratio.

Aromatic plants can express some immunomodulatory properties by improving phagocytosis, and they can modulate immunoglobulin secretion and enhance lymphocyte expression (Christaki et al., 2020). Absorbed phytogenic compounds might produce changes in blood immunological parameters by increasing peripheral blood cell immune response and concentration of immunoglobulins, and while unabsorbed they may stimulate intestinal immune function (Bozkurt & Tüzün, 2020).

The phenolic compounds (e.g. thymol and carvacrol) are mainly effective against intestinal colonisation by undesirable pathogenic bacteria, and could positively affect the growth of beneficial bacteria such as lactobacilli. Moreover, these substances are effective against *Eimeria* infection and other gut parasite expression (Christaki et al., 2020).

The objectives of this study were to evaluate the effects of sustained application of thymol and its withdrawal on thymol absorption from the gastrointestinal tract and excretion from the body, and to identify the relation between its bioavailability and the intestinal wall morphology, microbiota of the large intestine and immune response, all considering the specific digestive processes in rabbits.

2 | MATERIAL AND METHODS

2.1 | Animals, experimental design and animal care

A total of 48 rabbits of both sexes (meat line M9) at 5 weeks of age were randomly divided into two dietary treatment groups (C/control, T/thymol addition) of 24 animals in each with six replicates (two cages-one cage with two rabbits/one replicate). The rabbits were fed with 0.025% thymol addition for 3 weeks and for the following week the thymol was withdrawn, so finally the experiment lasted 1 month. The animals could feed ad libitum and had free access to drinking water. All experimental wire-net cages (61 cm × 34 cm × 33 cm) were kept in rooms with automatic temperature and humidity control by means of digital thermograph (22 ± 4°C, 70 ± 5%). A lighting regimen of 16 h light and 8 h dark was applied during the whole experiment. Eight rabbits (6 male, 2 female) at the age of eight (with thymol) and nine (without thymol) weeks were stunned using electronarcosis (50 Hz, 0.3 A/rabbit for 5 s), immediately hung by the hind legs on the processing line and quickly bled by cutting the jugular veins and the carotid arteries.

2.2 | Experimental diet and thymol stability in feed

The animals were fed a diet appropriate to the requirements of growing rabbits in pellet form (3.5 mm average size). The ingredients and chemical composition of the diet are presented in Table 1. To determine the crude protein (CP), ash, acid detergent fibre (ADF) and dry matter (DM) contents, the Association of Official Analytical Methods (AOAC, 2005) protocols were used. Neutral detergent fibre (NDF) was analysed according to Van Soest et al. (1991). Thymol (≥99.9%; Sigma-Aldrich) in white powder form was added to the basal diet at 0.025% concentration. Stability of thymol in the feed was analysed every week during the thymol addition period by means of high-performance liquid chromatography (HPLC) according to the modified method of Pisarčíková et al. (2017). The obtained thymol concentrations in weeks one, two and three were 189.5, 168.1 and 203.7 µg/g DM respectively, which confirmed its relative stability in the feed.

2.3 | Antioxidative capacity of thymol

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity as well as the Trolox equivalent capacity (TEAC) of pure thymol and the experimental feed was examined. The DPPH was determined using the spectrophotometric method described by Okada and Okada (1998). The basic principle of this method is the reduction of alcoholic DPPH solution in the presence of antioxidant, which serves as hydrogen donor. The result is normally expressed using the IC_{50} value, defined as the concentration of thymol which scavenges 50% of DPPH–free radicals.

The Trolox equivalent capacity (TEAC) was determined according to the method described by Karamác et al. (2020) using 2,2'-Azinobis-(3- Ethylbenzthiazolin-6-Sulfonic Acid (ABTS •+) decolorisation assay. The results were expressed as µmol Trolox equivalent (TE) per g of thymol or feed.

2.4 | Health status and sampling

Body weight (BW) and feed intake (FI) were recorded individually once a week. The average daily FI, average daily weight gain (ADG) and feed conversion ratio (FCR) were calculated at the end of the trial (at 8 and 9 weeks of age). Mortality was recorded daily throughout the experimental periods.

Tissues, faeces and blood (n = 8) were sampled on days 21 and 28 of the experiment. Blood for analysis of thymol and phagocytic activity was collected from the marginal ear vein (*vena auricularis*) before electronarcosis. Plasma for thymol analysis was obtained after centrifugation of blood at 1180 × g for 15 min. Samples of plasma, small intestine wall, caecal and caecal appendix content and hard faeces (freshly voided, collected using nets mounted under the cages) for thymol analyses were immediately frozen in liquid nitrogen and stored at −70°C until analysed. Dry matter of small intestinal wall, caecal and caecal appendix content and faeces was acquired by drying samples at 135°C to constant weight (AOAC, 2005). The same eight rabbits per treatment were used for gut morphometric and immunoglobulin A analyses. Part of the proximal small intestinal segment (jejunum, approx. 10 cm) was excised. To remove all intestinal content, it was flushed with 0.9% saline (approx. 5 cm was immediately frozen for IgA), then fixed in 4% neutral formalin solution and subsequently submitted for morphometric analysis. To test the microbiota, samples (approximately 1 g) of caecal appendix and caecal content were collected from the same animals, and faeces (n = 6, one sample from one replicate) on the same days of age.

TABLE 1 Ingredients and chemical composition (g/kg DM) of experimental diets (dry matter-DM basis)

Ingredients	g/kg DM	Composition	g/kg DM
Dehydrated lucerne meal	324.35	Dry matter (g/kg)	900.98
Dry malting sprouts	135.15	Organic compounds	749.42
Oats	117.13	Nitrogen free extract	400.26
Wheat bran	81.09	Neutral detergent fibre	317.95
Barley	72.08	Acid detergent fibre	187.49
Extracted sunflower meal	49.55	Crude fibre	160.23
Extracted rapeseed meal	49.55	Crude protein	159.12
Dried distillers grain with solubles	45.05	Cellulose	147.00
Premix[a]	15.32	Hemicellulose	130.45
Limestone	9.01	Starch	119.88
Sodium chloride	2.70	Ash	62.35
Total	900.98	Fat	29.80
		Metabolic energy, MJ/kg	9.91

Abbrevation: DM, dry matter.

[a]The vitamin–mineral premix provided per kg of complete diet: Retinyl acetate 5.16 mg, Cholecalciferol 0.03 mg, Tocopherol 0.03 mg, Thiamin 0.8 mg, Riboflavin 3.0 mg, Pyridoxin 2.0 mg, Cyanocobalamin 0.02 mg, Niacin 38 mg, Folic acid 0.6 mg, Calcium 1.8 mg, Iron 70 mg, Zinc 66 mg, Copper 15, Selenium 0.25 mg.

2.5 | Analysis of thymol in plasma, small intestinal wall, caecal content and faeces

Detection of thymol in samples of plasma, intestinal wall, caecal content and faeces was performed using headspace solid-phase microextraction followed by gas chromatography coupled with mass spectrometry (GC/MS). Detection using total-ion current (TIC) trace and quantification by selected-ion monitoring (SIM) were carried out using GC/MS type HP 6890 GC coupled with a 5972 quadrupole-mass selective detector (Agilent Technologies GmbH). Detection of thymol was confirmed by comparing its specific mass spectrum and retention time with those of the authentic reference compound. Additionally, the Kovats index was calculated. Enzyme β–Glucuronidase Helix pomatia Type HP-2 (aqueous solution, ≥100,000 units/ml, Sigma-Aldrich) was added to samples to cleave thymol from its glucuronide and sulphate to obtain the total amount of thymol (Oceľová et al., 2016).

2.6 | Gut morphology investigation

The proximal part of small intestinal wall was routinely embedded in paraffin, sectioned at 5 μm thickness and mounted on glass slides. Ten serial sections in total were prepared from each intestinal sample, stained with haematoxylin/eosin and observed under a light microscope using the method described by Žitňan et al. (2008). The evaluated indices were: villi height (Vh, villi tip to base of villi), crypt depth (Cd, based of villi to bottom of crypt) and the ratio of villi height to crypt depth (Vh/Cd) (Trebušak et al., 2019).

2.7 | Immunoglobulin A in small intestinal wall and phagocytic activity in blood

For quantitative measurement of immunoglobulin A (IgA) in the intestinal wall, the competitive inhibition enzyme immunoassay technique was used (Cusabio). Intestinal wall samples were prepared using the method described by Nikawa et al. (1999).

The direct microscopic counting procedure, using the yeast-cell method, was used for phagocytic activity (PA) analysis in blood (Šteruská, 1981). Blood smears stained with May-Grünwald and Giemsa-Romanowski stains were used for calculating the number of white cells containing at least three engulfed particles per 100 white cells (monocytes/granulocytes).

2.8 | Microbial evaluation

The samples of faeces, caecal and caecal appendix content (1 g) were treated using the standard microbiological dilution method proposed by the International Organization for Standardization (ISO). The appropriate dilutions in Ringer solution (pH 7.0; Oxoid Ltd.) were cultivated as described by Pogány Simonová et al. (2020). Cultivation was performed at 30°C and/or 37°C for 24–48 h, depending on the bacterial genera. The bacterial counts were expressed in log 10 of colony-forming units per gram ($\log^{10}$ CFU/g ± SEM). Randomly picked representatives of selected bacterial groups were confirmed using the MALDI-TOF identification system (Bruker Daltonics).

2.9 | *Eimeria* sp. oocyst counting

Faecal samples were examined for *Eimeria* oocysts, collected from six animals per treatment group in the morning on experimental days 21 and 28, and stored at 4°C until examination. The flotation method was used for quantitative evaluation of oocysts according to McMaster (1986). Oocyst counts were expressed per gram of faecal sample (OPG).

2.10 | Ethical issues

This experiment was carried out at the experimental rabbit facility of the National Agricultural and Food Centre, Research Institute for Animal Production, Nitra, Slovakia. The protocol was approved by the Institutional Ethics Committee, and the State Veterinary and Food Office of the Slovak Republic approved the experimental protocol (4047/16-221).

2.11 | Statistical analysis

The statistical analyses were performed using GraphPad Prism version 5.0 for Windows, GraphPad Software, www.graphpad.com. The Kolmogorov–Smirnov test evaluated normality or non-normality of distribution. Statistical analysis of the results used analysis of variance as a 2 × 2 factorial design that represents two main factors: time of measurements (8 and 9 weeks) and treatment (with and without thymol). Three main objectives were examined: the effect of time, the effect of thymol and the interaction between time and thymol addition. Differences between diets with and without thymol addition were analysed by two-way analysis of variance (ANOVA). When interaction between time and treatment was statistically significant, the simple Mann–Whitney *U* test was performed. For comparison of thymol concentration between plasma, intestinal wall, caecal content and faeces Kruskal–Wallis test with post hoc Dunn´s Multiple Comparison test was used. Correlations of thymol concentration between plasma and intestinal wall, caecal content, faeces; intestinal wall and caecal content, faeces; caecal content and faeces were analysed using nonparametric Spearman's Rank Correlation and expressed as Spearman's correlation coefficient (r_s). Results are presented as the mean ± standard error of mean (SEM). Significant differences were considered at $p < 0.05$.

3 | RESULTS

All data was not accepted as parametric data.

3.1 | Thymol in plasma, small intestinal wall, caecal content and faeces

Thymol content in faeces ($p < 0.001$) and caecal content ($p < 0.05$) revealed significantly higher concentrations in comparison with plasma during the thymol addition and withdrawal (2442 ± 451.4, 881.9 ± 231.3 ng/g DM and 46.2 ± 28.4 ng/ml, respectively and 149.5 ± 40.54, 45.76 ± 12.44 ng/g DM and 2.73 ± 0.45 ng/ml, respectively). It is important to notice, that after withdrawal was thymol detected only in traces amount except the faeces (Figure 1). Only the correlation between thymol content in plasma and intestinal wall was observed on statistical significant differences during the period of thymol addition ($r_s = -1.0$, $p < 0.01$).

3.2 | Antioxidative capacity of thymol

Approximately 34.48 µg/g of thymol (35.50 µg/g, feed) trapped 50% of DPPH free radicals. The oxygen radical absorbance capacity value of thymol was 760 µmol TE/g and thymol's free-radical scavenging ability was also confirmed after its mixing into the feed (740 µmol TE/g of feed).

3.3 | Growth performance

Throughout this experiment most animals were in good health and slaughter weight was higher at 9 weeks than 8 weeks of age ($p < 0.001$). Other parameters of growth performance (ADG, FCR) were not significantly influenced by none of factors (Table 2). Three animals from the control and two from the experimental group died during the whole experiment.

3.4 | Gut morphology

Villi height to crypt depth ratio was significantly higher during the thymol addition and remained so after its withdrawal ($p < 0.05$, Table 3).

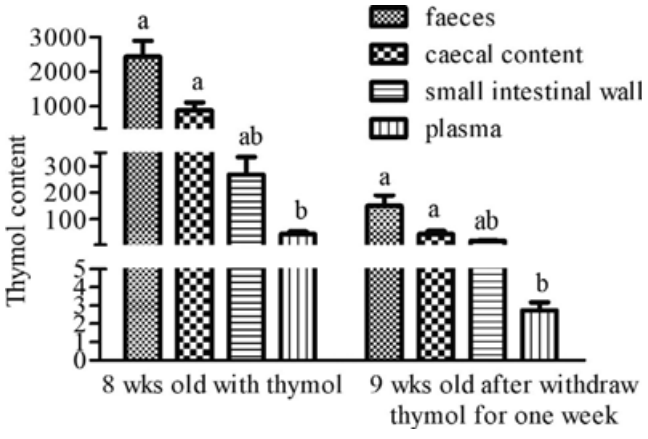

FIGURE 1 Thymol in faeces, caecal content, small intestinal wall (ng/g DM) and in plasma (ng/ml) during thymol addition and after withdrawal. Data are presented as mean ± standard error of mean (SEM). [a, b] Means which do not share a common superscript are significantly different (p < 0.05). DM, dry matter

TABLE 2 Effects of thymol on growth performance parameters of rabbits

Parameter	Weeks	Experimental group		SEM	*p*-value		
		Control	Thymol		Treatment	Time	Interaction
Slaughter weight (g)	8	2044	1965	32.21	0.6872	<0.0001	0.0819
	9	2671	2796	48.04			
ADG (g/day/rabbit)	8	41.59	41.32	1.54	0.6250	0.4174	0.5547
	9	37.90	40.73	1.56			
FCR	8	3.47	3.58	0.28	0.9327	0.7904	0.8608
	9	3.66	3.62	0.33			

Note: 8 weeks old with thymol addition, 9 weeks old after withdraw thymol for 1 week. Data are presented as mean ± standard error of mean (SEM). Abbrevations: ADG, average daily weight gain; FCR, feed conversion ratio.

3.5 | Immunoglobulin A in small intestinal wall and phagocytic activity in blood

There was interaction between studied factors on IgA and PA in this study ($p < 0.05$). The IgA was high in the groups fed diets with thymol comparing to control group at 8 weeks of age. However, IgA was similar between experimental group at 9 weeks of age which none of main factor was statistical significant differences ($p > 0.05$). In contrast, both main factors were statistical significant differences ($p < 0.001$) which the treatment group was higher than control group. In addition, the PA was lower in both groups in 9 weeks old but the PA in treatment group still higher than the control group at 9 weeks old (Table 3).

3.6 | *Eimeria* sp. oocysts in faeces

The none of oocytes was presented in the thymol group, while the control group without supplementation was found oocytes in high number ($p < 0.05$). However, the oocytes were found again in both groups at 9 weeks old (Table 3).

3.7 | Microbial evaluation

Enterococcus sp. in faeces was lower in 9 weeks old rabbits comparing to 8 weeks old ($p < 0.05$). Lactic acid bacteria (LAB) in caecal appendix ($p < 0.01$) and faeces ($p < 0.05$) were higher in the treatment groups, in faeces also higher in the 9 weeks old ($p < 0.05$). Coagulase-negative staphylococci (CoNS) in all collected site was lower at 9 weeks old comparing to 8 weeks old ($p < 0.05$) and in faeces of groups fed thymol was higher than the control group ($p < 0.05$). Coagulase-positive staphylococci (CoPS) in caecal appendix was lower in the treatment group ($p < 0.01$). The statistical significance on interaction between studied factors without differences on main effects was presented for CoPS in faeces. The treatment group was lower on CoPS in faeces at 8 weeks old, however, was higher in this group at 9 weeks old. None difference was found between time and groups in all collected sites for Coliform bacteria ($p < 0.05$, Table 4).

4 | DISCUSSION

Even though plant extracts and essential oils have shown promising results as growth promoters in many animal species, we did not confirm any stimulating effect of thymol on weight gain or feed conversion ratio in our study. Following on from the results of Fekete and Lebas (1983) and Dalle Zotte et al. (2013), no improvement in live performance of rabbits after dietary supplementation with thyme has yet been confirmed. Erdelyi et al. (2008) argued that various herbs and spices have little positive effect on the growth of rabbits probably due to their specific digestive physiology.

Thymol absorption in the small intestine was relatively high due to the good blood supply to the intestinal wall. The high absorption rate of thymol in our experiment was confirmed by the negative correlation between plasma and intestinal wall during the thymol addition. The processes running in the intestinal wall are crucial not only for the absorption but also biotransformation of thymol. After biotransformation into enterocytes, thymol or its metabolites can be transported back into the intestinal lumen or are distributed within the organism through the blood and systemic circulation. Glycosidases, glucuronidases and sulfatases in the intestinal microflora are able to release the parent compounds from their metabolites, which can then be excreted in this form in the faeces. The biotransformation processes are repeated until the thymol is eliminated from the body (Bacova et al., 2020; Placha et al., 2019). As thymol metabolites can be converted back into parental compounds by microbial enzymes in the caecum, we assume that this process together with consumption of cecotrophs containing thymol (metabolised or non-metabolised) could explain the higher amount of thymol in the caecal content. As cecotrophy is the process of re-digestion and absorption of previously undigested nutrients, this together with processes of biotransformation could explain our detection of thymol in the faeces also after its withdrawal. Moreover, as described in the previous study by Oceľová et al. (2016), there is a possibility of thymol binding to the surface of red blood cells, with subsequent recirculation in the organism and release into the intestine as a result of biotransformation.

In general, the term "gut health" represents interaction between the microbiome, the intestinal wall barrier and physiological and

TABLE 3 Effects of thymol on small intestinal wall morphometric indices, immunoglobulin A in intestinal wall, phagocytic activity in blood and *Eimeria* sp. oocytes in faeces of rabbits

		Experimental group			*p*-value		
Parameter	**Week**	**Control**	**Thymol**	**SEM**	**Treatment**	**Time**	**Interaction**
Vh (μm)	8	693.40	700.60	3.48	0.1312	0.4784	0.9915
	9	690.00	697.30	3.15			
Cd (μm)	8	181.60	179.90	1.41	0.2903	0.1831	0.7941
	9	179.30	176.50	1.50			
Vh/Cd	8	3.82	3.90	0.02	0.0038	0.1211	0.6478
	9	3.85	3.95	0.02			
IgA (μg/g)	8	10.42	22.42	2.04	0.0862	0.8503	0.0046
	9	17.50	14.38	2.32			
PA (%)	8	40.13	66.13	3.59	<0.0001	<0.0001	<0.0001
	9	40.88	47.38	1.25			
Eimeria sp. (OPG)	8	2383.00	ND	1962.00	0.0058	0.8710	0.5654
	9	1108.00	1392.00	363.70			

Note: 8 weeks old with thymol addition, 9 weeks old after withdraw thymol for 1 week. Data are presented as mean ± standard error of mean (SEM).
Abbrevations: Vh, villi height; Cd, crypt depth; Vh/Cd, villi height to crypt depth ratio; IgA, immunoglobulin A; PA, phagocytic activity; OPG, oocytes per gram faeces; ND, not detected .

immune components, which allow different animals to cope with internal and external stressors (Artuso-Ponte et al., 2020).

In the present study, dietary supplementation with thymol as well as its withdrawal significantly increased the villi height/crypt depth ratio and the villi height numerically. Bozkurt et al. (2012) suggested the protective properties of bioactive compounds on the intestinal villi, particularly carvacrol, thymol and 1,8-cineol, which they explained in terms of acceleration in the renewal rate of mature enterocytes as a consequence of the sparing effect of these compounds against oxidative damage. Based on previous results from Bacova et al. (2020), who confirmed the alleviation of oxidative damage by means of thymol addition to the rabbit diet, and based on thymol detection in the intestinal wall in our study, we assume it has beneficial effect on enterocytes. In their study Rubió et al. (2014) attributed important antioxidant activity to the conjugated form of thymol, thymol sulphate. Thymol metabolites such as thymol sulphate and thymol glucuronide were detected in the duodenal wall of poultry by Pisarčíková et al. (2017). We can assume that the presence of these metabolites in the intestinal wall of rabbits in our experimental study could have a sparing effect against oxidative damage of intestinal cells. Unfortunately, this is only a hypothesis, because it has not been confirmed so far whether these metabolites are active or inactive.

The secretory form of IgA is synthesised by plasma cells in the lamina propria and is translocated through intestinal epithelial cells. IgA is bound to the receptor at the basolateral surface of enterocytes and subsequently crosses the epithelial layer (Macpherson et al., 2001). Based on our detection of thymol in the intestinal wall and its antioxidant effects as confirmed in previous studies by Placha et al. (2019) and Bacova et al. (2020), we assume that the higher amount of IgA detected in the intestinal wall is the result of thymol sparing effects on enterocytes and consequently of larger amounts of IgA bonding on their surface. Plasma cells which produce IgA are developed from antigen-stimulated B cells (Tizard, 2004). According to Macpherson et al. (2001), B cells reach intestinal mucosa sites via the lymphatic system and arterial blood. If thymol can bind to erythrocytes as described Rubió et al. (2014) and Oceľová et al. (2016), its amount in the blood should be higher than in plasma. For this reason and from our results suggesting effective absorption of thymol, we can assume that the amount of thymol in our experiment was sufficient to protect the B cells against oxidative damage, and consequently to allow secretion of higher amounts of IgA, which is then translocated through the epithelial cells of the intestinal wall to the intestinal lumen. Activity of phagocytic cells such as neutrophils, monocytes and macrophages plays an important role in innate immunity and can be modulated by thymol (Chauhan et al., 2014). Muller et al. (1989) confirmed that thymol is able to affect the intracellular mechanism of macrophages by increasing membrane fluidity and activation of membrane proteins, and consequently effectively boosts phagocytosis. In our experiment, the bonded thymol probably circulated by means of systemic circulation and was released also after its withdrawal, which may explain the increase in phagocytic activity during thymol addition as well as after its withdrawal.

According to the findings of Dorman and Deans (2000), thymol as the main compound of *T. vulgaris* essential oil expressed greater inhibitory activity against Gram-positive than Gram-negative bacteria. Even though we expected the inhibitory effect of thymol on pathogenic bacteria in our study, we could not confirm this expectation either during thymol addition or after its withdrawal. Surprisingly, only beneficial LAB increased significantly in the caecal appendix during the period with thymol addition (Table 3). The same tendency to colonise the rabbit gut with beneficial bacteria after supplementation

TABLE 4 Effect of thymol on bacterial counts in caecal appendix, caecal content and faeces of rabbits (log 10 CFU/g)

		Experimental group			*p*-value		
Parameter	**Weeks**	**Control**	**Thymol**	**SEM**	**Treatment**	**Time**	**Interaction**
Enterococcus sp.							
Caecal appendix	8	2.03	1.26	0.32	0.1842	0.1210	0.3351
	9	1.18	1.06	0.09			
Caecal content	8	1.19	1.20	0.12	0.7586	0.0539	0.7150
	9	0.99	0.97	0.01			
Faeces	8	5.65	5.56	0.13	0.3202	0.0143	0.2007
	9	4.46	5.16	0.27			
LAB							
Caecal appendix	8	4.26	5.67	0.28	0.0033	0.5610	0.3285
	9	4.39	5.14	0.26			
Caecal content	8	4.43	4.38	0.17	0.2259	0.7845	0.1800
	9	4.03	4.99	0.33			
Faeces	8	6.12	6.14	0.11	0.0385	0.0406	0.0493
	9	6.13	6.81	0.14			
CoNS							
Caecal appendix	8	3.30	3.88	0.29	0.4418	0.0069	0.3223
	9	2.67	2.60	0.08			
Caecal content	8	3.35	4.20	0.25	0.2204	0.0271	0.1783
	9	3.04	3.00	0.21			
Faeces	8	4.60	5.03	0.11	0.0193	0.0036	0.4507
	9	3.60	4.41	0.25			
CoPS							
Caecal appendix	8	4.19	3.03	0.33	0.0097	0.0636	0.0631
	9	1.82	2.04	0.09			
Caecal content	8	2.81	3.55	0.32	0.4390	0.2434	0.3888
	9	2.67	2.63	0.30			
Faeces	8	5.87	5.21	0.19	0.2953	0.4069	0.0022
	9	4.68	5.94	0.27			
Coliform bacteria							
Caecal appendix	8	2.95	3.57	0.44	0.6780	0.8177	0.5994
	9	3.14	3.07	0.46			
Caecal content	8	2.26	3.06	0.32	0.0839	0.2214	0.9869
	9	1.68	2.50	0.33			
Faeces	8	5.01	5.70	0.25	0.0561	0.5286	0.7169
	9	4.60	5.59	0.35			

Note: Data are presented as mean ± standard error of mean (SEM). 8 weeks old with thymol addition, 9 weeks old after withdraw thymol for 1 week.
Abbrevations: CoNS, coagulase-negative staphylococci; CoPS, coagulase-positive staphylococci; LAB, lactic acid bacteria; sp., species.

of thyme essential oil in their diet was demonstrated by Placha et al. (2013). Rhouma et al. (2018) also found a stimulatory effect of thymol on LAB in the distal gut, which is beneficial in preventing the proliferation of undesirable microorganisms. Based on Iqbal et al. (2020), the strong antioxidant properties of thymol could protect the intestinal epithelial cells and prevent inflammation, resulting in suppression of pathogenic bacteria on the one hand and supporting beneficial bacteria such as LAB on the other. Due to their low bioavailability, up to 90% of phenolic compounds enter the colon unaltered and interact with colonic bacteria (Espín et al., 2017). We detected approximately 5-times higher amounts of thymol in the faeces than in the intestinal wall during thymol addition. From this, we assume that thymol was present in adequate amounts in the caecal appendix as well, and was able to affect the microbiota in that area. Even so, we have to bear in mind that the knowledge about the bacterial composition in the rabbit caecal appendix is very scarce,

and further studies are surely needed to monitor microbial changes after various feed additives administration (Pogány Simonová et al., 2020). Increased concentration of microbiota, particularly of pathogenic Gram-positive bacteria like CoPS in the faeces after thymol withdrawal, indicate that their concentration during this period was unable to exert such antimicrobial effect as during the thymol addition (Table 4). Even though phenolic compounds are able to improve gut health due to their strong antioxidant and antimicrobial potential, further studies are needed to understand how the processes of thymol biotransformation in the gastrointestinal tract can affect the gut microbiota, because of the specificities of rabbit digestion.

Idris et al. (2017) suggested that compounds of essential oils are able to directly affect the parasitic metabolism by altering the permeability of cytoplasmic membranes or indirectly by enhancing the immune response and antioxidant defence system. Giannenas et al. (2003) and Küçükyilmaz et al. (2012) found that thymol and carvacrol reduced the number of oocytes in poultry faeces. Other studies have explained the mode of action of thymol and carvacrol in terms of destruction of the sporozoite membrane (Bozkurt et al., 2013). We confirm the anticoccidic effects of thymol in this experiment, and we agree with the conclusion of Felici et al. (2020) that its mode of action needs to be elucidated in the future.

5 | CONCLUSIONS

Dietary supplementation with thymol did not show any stimulating effect on growth performance. Thymol was sufficiently absorbed from the rabbit intestine and was also detected in their faeces even after its withdrawal from the diet as a consequence of caecotrophy, a nutritional characteristic specific for rabbit digestive processes, and of the biotransformation processes of thymol. Sufficient absorption of thymol and its bioavailability in the intestinal wall positively affected the villi height/crypt depth ratio and demonstrated beneficial effect on enterocytes. Administration of thymol at this concentration induced an immune response by stimulating the production of IgA in the intestinal wall and activity of phagocytic cells in the peripheral blood and expressed anticoccidic effect. Thymol did not reduce pathogenic bacteria, but was able to increase the number of beneficial LAB in the caecal appendix during its addition to the diet. As detailed information on thymol metabolism within the rabbit organism is lacking, further research should be done to establish the relation between its concentration and biological role to optimise its effect on rabbits´ health, before recommending the application of thymol to farm animals generally.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Iveta Placha involved in conceptualisation, methodology, validation, investigation, resources, data curation, funding acquisition, writing–original draft, visualisation, and project administration. Kristina Bacova involved in formal analyses and writing–original draft. Karin Zitterl-Eglseer involved in formal analyses, resources, and writing–review and editing. Andrea Laukova involved in formal analyses, resources, and data curation. Lubica Chrastinova involved in resources and data curation. Michaela Madarova involved in formal analyses. Rudolf Zitnan involved in formal analyses and resources. Strkolcova Gabriela involved in formal analyses and resources.

ANIMAL WELFARE STATEMENT

The authors confirm that the ethical policies of the journal have been adhered to, and the appropriate ethical approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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