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To cite this article: Kristina Bacova, Karin Zitterl-Eglseer, Andrea Laukova, Lubica Chrastinova, Sona Gancarcikova, Rudolf Zitnan, Margareta Takacsova, Drahomira Sopkova, Zuzana Andrejckakova, Monika Pogany Simonova, Radoslava Kristofova, Zuzana Formelova & Iveta Placha (2023) Effect of thymol and Enterocin M administration on biochemical, antioxidant and immunological parameters, small intestinal morphology and microbiota in rabbits., *Italian Journal of Animal Science*, 22:1, 972-981, DOI: [10.1080/1828051X.2023.2257732](https://doi.org/10.1080/1828051X.2023.2257732)

To link to this article: <https://doi.org/10.1080/1828051X.2023.2257732>



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Published online: 22 Sep 2023.



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RESEARCH ARTICLE



Effect of thymol and Enterocin M administration on biochemical, antioxidant and immunological parameters, small intestinal morphology and microbiota in rabbits.

Kristina Bacova^{a,b}, Karin Zitterl-Eglseer^c, Andrea Laukova^a , Lubica Chrastinova^d, Sona Gancarcikova^e , Rudolf Zitnan^d, Margareta Takacsova^a , Drahomira Sopkova^f, Zuzana Andrejczakova^f , Monika Pogany Simonova^a , Radoslava Kristofova^{a,b}, Zuzana Formelova^d and Iveta Placha^a 

^aCentre of Biosciences of the Slovak Academy of Sciences, Institute of Animal Physiology, Oddelenie fyziologie traviaceho traktu, Kosice, Slovakia; ^bUniversity of Veterinary Medicine and Pharmacy, Kosice, Slovakia; ^cDepartment für Nutztier und öffentliches Gesundheitswesen, Institute of Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine Vienna, Vienna, Austria; ^dNational Agricultural and Food Centre, Nitra-Luzianky, Slovakia; ^eKatedra mikrobiologie a imunologie, University of Veterinary Medicine and Pharmacy, Kosice, Slovakia; ^fKatedra biologie a fyziologie, University of Veterinary Medicine and Pharmacy, Kosice, Slovakia

ABSTRACT

To find natural feed additives with a beneficial effect on rabbit health, thymol alone and in combination with Enterocin M were administered in drinking water for 42 days (35–77 days of age). A total of 48 rabbits based on their weight were randomly divided into four experimental groups: C – control (basal diet), T – thymol (250 mg/L), E – Enterocin M (Ent M) (50 µL/animal/day), T + E (thymol with Ent M). Ent M ($p < .05$) and thymol ($p < .01$) separately decreased malondialdehyde in the liver. Thymol separately and in combination significantly increased phagocytic activity in the blood ($p = .0051$) and lactic acid in the caecum ($p = .0142$) and decreased coagulase-positive staphylococci in the caecum ($p = .0329$). Ent M separately and in combination increased immunoglobulin A content in the jejunal wall ($p = .002$) and decreased coliform bacteria in faeces ($p = .0002$). Thymol and Ent M application separately or in combination improved the antioxidant and immune response of rabbits and demonstrated an antibacterial effect.

HIGHLIGHTS

- Thymol demonstrated antibacterial effect against coagulase-positive staphylococci (CoPS) the in caecum, the formation of malondialdehyde (MDA) as a product of lipid peroxidation in liver, and a stimulation effect on fermentation the processes in the caecum and on phagocytic activity in blood.
- Ent M decreased coliform bacteria in faeces and stimulated the production of IgA in jejunum.
- Ent M together with thymol improved the structure of the jejunal wall and liver function.

ARTICLE HISTORY

Received 29 June 2023
Revised 6 September 2023
Accepted 6 September 2023

KEYWORDS

Natural additives; oxidative stress; gut health

Introduction

After the European Union banned antibiotic growth promoter, natural additives like prebiotics, probiotics, postbiotics and medicinal plants have been investigated more intensively. The main reason for this prohibition was the development of resistant bacterial strains and transfer resistance to other strains of bacteria populations (Skoufos et al. 2016). According to EU legislation, natural feed additives are substances with a favorable effect on animal nutrition and performance as well as the production of safe food

products to satisfy the increasing consumer demand for safe rabbit food (Placha et al. 2022a).

As plant secondary metabolites show noteworthy antimicrobial, antioxidant, and immunostimulant properties, animal nutritionists, veterinarians and animal owners have begun to express interest in veterinary herbal medicine (Mertenat et al. 2020). *Thymus vulgaris* has a long history of use for different medicinal purposes, mainly due to thymol, which is the main compound with a phenolic structure (Salehi et al. 2018). One crucial aspect of the beneficial effect of thymol

CONTACT Iveta Placha  placha@saske.sk

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and other plant compounds is the amount of them present in the gut as a result of being released from feed. Bacova et al. (2021) and Placha et al. (2022b) confirmed intensive absorption of thymol from the gastrointestinal tract after its sustained oral administration in rabbits. They demonstrated its beneficial effect on the intestine structure, antioxidants and immune response of the rabbit organism and gut microflora. Placha et al. (2022c) emphasised that more studies are needed to precisely understand thymol biological activity in order to achieve the required beneficial effect in the animal organism.

The positive effect of probiotics and postbiotics, including bacteriocins, which are antimicrobial proteins produced mostly by lactic acid bacteria, on rabbit health parameters were presented by Pogány Simonová et al. (2020a, 2020b) and Lauková et al. (2012, 2016). Those authors demonstrated the positive impact on intestinal microbiota balance and jejunal morphological parameters and confirmed their immuno-stimulative effect in rabbits. Pogány Simonová et al. (2020a, 2020b) investigated the effect of Ent M in combination with sage extract on rabbit health and determined the antibacterial effect of both additives in the gastrointestinal tract along with the optimisation of the investigated biochemical parameters and improvement of jejunal morphological parameters, mostly by Ent M.

In our previous studies we confirmed the beneficial antioxidant and immunostimulant effect and the improvement of intestinal wall structure after thymol administration in rabbits (Bacova et al. 2020; Placha et al. 2022b). As was mentioned in our last study (Placha et al. 2022c), only a few studies on thymol bioavailability in different tissues have been conducted to date. Based on the above-mentioned knowledge, we decided to apply thymol alone and in combination with Ent M to compare their effect on selected biochemical, antioxidant and immunological parameters as well as jejunum morphology and microbiota.

Materials and methods

Animals, experimental design and animal care

A total of 48 rabbits of both sexes (meat line M91) weaned at five weeks of age were randomly divided into four experimental groups: C – control (basal diet, average weight 1000.0 ± 48.5 g), T – thymol (average weight 1030.0 ± 54.8 g), E – Enterocin M (Ent M, average weight 1030.0 ± 73.5 g), T + E (thymol in combination with Ent M, average weight 1012.0 ± 74.6 g), each with 12 animals (10 males, 2 females; two rabbits/cage). The animals were fed *ad libitum* and had free access to drinking

water. All the experimental wire-net cages (61 cm x 34 cm x 33 cm) were kept in rooms with automatic temperature and humidity control by means of a digital thermograph (22 ± 4 °C, $70 \pm 5\%$). A lighting regimen of 16 h light and 8 h dark was applied throughout the experiment. Animals were in good health during the whole experimental period, and the average final live weights of the rabbits were 2504.0 ± 82.3 g (C), 2446.0 ± 65.8 g (E), 2528.0 ± 142.4 g (T) and 2484.0 ± 85.6 g (E + T). Eight randomly selected rabbits (males) from each group at the age of eleven weeks (the experiment lasted 42 days) 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.

Experimental diets

The animals were fed a diet appropriate to the requirements of growing rabbits in pellet form (KKZK, Liaharensky podnik Nitra, Slovakia). The ingredients and chemical composition of the diet are presented in Table 1. The Association of Official Analytical Chemists (AOAC, 2005) methods were used to determine the proportion of crude protein, ash and dry matter; neutral and acid detergent fibres were analysed according to Van Soest et al. (1991). The rabbits in group T received thymol ($\geq 99.9\%$, Sigma Aldrich, St. Louis, USA) dissolved in drinking water in a concentration of 0.025% (25 mg/100 mL)/animal/day. The rabbits in group E received Ent M (produced by *Enterococcus faecium* AL41/CCM 8558 strain), prepared according to Mareková et al. (2007) in a dose of 50 µL/animal/day (with activity of 25,600 AU/mL, in a concentration of 0.4 g/L). The activity of the applied Ent M was tested with the agar spot test according to De Vuyst et al. (1996) against the principal indicator strain *E. avium* EA5 (isolated from piglet faeces in our laboratory). The rabbits in group T + E received a combination of thymol (25 mg/100 mL) dissolved together with Ent M (50 µL/animal/day, 25,600 AU/mL, 0.4 g/L) in drinking water (100 mL). The additives were applied to rabbits every morning firstly in volume 100 mL during all experimental period (42 days) provided through nipple drinkers and after consuming this volume the rabbits had access to water *ad libitum*.

Sampling

Before electronarcosis, blood samples were collected from the marginal ear vein (*Vena auricularis*). Blood for biochemical analyses was collected into dry

Table 1. Ingredients and chemical composition (g/kg DM) of experimental diets.

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 fiber	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 DM	9.91

^aThe vitamin-mineral premix provided per kg of complete diet: Retinyl acetate 5.16 mg, Cholecalciferol 0.03 mg, Tocopherol 0.03 mg, Thiamine 0.8 mg, Riboflavin 3.0 mg, Pyridoxine 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 mg, Selenium 0.25 mg.
DM: dry matter.

nonheparinized Eppendorf tubes and was left to clot in a standing position for approximately 2 h to obtain the serum, which was then separated by centrifugation at $1000 \times g$ for 15 min. Blood for analyses of the antioxidant parameters and thymol was collected into heparinised tubes, and plasma was obtained after centrifugation at $1180 \times g$ for 15 min.

Samples of blood, serum, plasma, jejunal intestinal segment with mucosa, muscle (*Longissimus thoracis et lumborum*, LTL), kidney and liver were immediately frozen in liquid nitrogen and stored at -70°C until analysed. Dry matter from the jejunum, muscle, liver and kidney was acquired by drying samples at 135°C to a constant weight (AOAC, 2005). The same eight rabbits per treatment were used for gut morphometric analyses. Part of the proximal 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 (microbiota + organic acids) were collected from the same animals. Faeces were collected using nets mounted under the cages ($n=8$).

Analysis of thymol in plasma, jejunal wall and muscle

Detection of thymol in samples of plasma, jejunal wall and muscle 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 a GC/MS type HP 6890 GC coupled with a 5972

quadrupole-mass selective detector (Agilent Technologies, Wilmington, DE, USA). 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. The selective ion mode was used for quantitation of thymol. The mass fragments m/z 135 and m/z 150, as well as m/z 107 and m/z 108, were monitored as characteristic for thymol and o-cresol (internal standard), respectively. Enzyme β -Glucuronidase Helix pomatia Type HP-2 (aqueous solution, $\geq 100,000$ units/mL, Sigma-Aldrich, St Louis, MO, USA) was added to the samples to cleave thymol from its glucuronide and sulphate to obtain the total amount of thymol (Ocelová et al. 2016).

Biochemical, antioxidant parameters and activity of lactate dehydrogenase in blood and tissues

Total proteins (TP), creatinine, urea, triglycerides, total cholesterol, alanine aminotransferase (ALT, EC 2.6.1.2.), aspartate aminotransferase (AST, EC 2.6.1.1.), gamma glutamyl transferase (GMT, EC 2.3.2.2.) and alkaline phosphatase (ALP, EC 3.1.3.1.) were analysed using a Dialab commercial kit (Czech Republic) and an ELLIPSE analyser (AMS, Italy).

The activity of glutathione peroxidase (GPx, EC 1.11.1.9) in blood was measured by monitoring the oxidation of NADPH at 340 nm in line with Paglia and Valentine (1967), using a commercial kit (Ransel, Randox, UK). Haemoglobin (Hb) content in blood was analysed using a commercial kit from Randox, UK. The samples of LTL muscle and liver for malondialdehyde (MDA) measurement and activity of lactate dehydrogenase (LDH, EC 1.1.1.27) were washed in buffered saline to remove excess blood and connective tissue. Samples for MDA analyses were homogenised with de-ionised distilled water and 50 μL of 7.2% butylated hydroxytoluene, and

for LDH activity in a cold buffer (0.05 mol/L Tris-HCl buffer, pH 7.3). The homogenates were subsequently centrifuged at $13,680 \times g$ at 4°C for 20 min. MDA concentrations in these tissues and plasma were measured using the modified fluorometric method of Jo and Ahn (1998). The enzyme activity of LDH was measured using a commercial diagnostic kit (Crumlin, Randox, UK) with an Alizé automatic biochemical analyser (Lisabio, Pouilly-en-Auxois, France) at 340 nm, as described by Andrejčáková et al. (2016). The protein concentration in muscle and liver was quantified using the spectrophotometric method published by Bradford (1976).

Gut morphology investigation

The proximal part of the jejunal organ was routinely embedded in paraffin, sectioned at $5\mu\text{m}$ 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 Zitnan et al. (2008). The evaluated indices were villus 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).

Immunoglobulin A in jejunal wall and phagocytic activity in blood

For quantitative measurement of immunoglobulin A (IgA) in the whole intestinal wall (with mucosa), the competitive inhibition enzyme immunoassay technique was used (Cusabio, Wuhan, China). 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).

Organic acid analyses

Lactic acid (g/100 g) and volatile fatty acid (VFA) values (acetic, propionic, butyric) were determined (mmol/L) using gas chromatography (Perkin Elmer gas chromatograph, USA) from samples of caecal content (15 g) using the method described by Pogány Simonová et al. (2020a, 2020b).

Microbial evaluation

The samples of faeces, caecal and appendix content (1 g) were treated using the standard microbiological dilution method proposed by the International Organisation for Standardisation (ISO). The appropriate dilutions in Ringer solution (pH 7.0; Oxoid Ltd., Basingstoke, Hampshire, England) were plated onto following media: M-Enterococcus Agar (NF-V04503, Difco Laboratories, Detroit, MI, USA) for enterococci, De Man-Rogosa-Sharpe agar (ISO 15214, Merck, Germany) for lactic acid bacteria (LAB), MacConkey agar (ISO 7402, Oxoid) for coliforms, Mannitol Salt Agar for coagulase-negative staphylococci (CoNS, ISO 6888), Baird-Parker agar enriched with egg yolk tellurite supplement (ISO 21527-1, Difco) for coagulase-positive staphylococci (CoPS). Cultivation was performed at 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 ($\log_{10} \text{CFU/g} \pm \text{SD}$). Randomly picked representatives of selected bacterial groups were confirmed using the MALDI-TOF identification system (Bruker Daltonics).

Statistical analysis

The statistical analyses were performed using GraphPad Prism version 9.5.1 for Windows, GraphPad Software, San Diego, California, USA, 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: Ent M (with and without) and thymol (with and without). Three main objectives were examined: the effect of Ent M, the effect of thymol and the interaction between Ent M and thymol addition. Differences between diets with and without additives were analysed by two-way analysis of variance (ANOVA). When the interaction between Ent M and thymol was statistically significant, the Kruskal-Wallis test with post hoc Dunn's Multiple Comparison test was used. For comparison of thymol concentration in plasma and jejunal wall between groups T and T+E, the simple Mann-Whitney U test was performed. Results are presented as the mean $\pm$ standard deviation (SD). Significant differences were considered at $p < .05$.

Results

Thymol in plasma, jejunal wall and muscle

Thymol content in jejunal wall was $147.6 \pm 79.9 \text{ ng/g DM}$ (T) vs $187.3 \pm 113.3 \text{ ng/g DM}$ (T+E, $p = 0.5350$).

Table 2. Effects of Enterocin M and thymol on biochemical parameters in serum of rabbits ($n = 8$).

Parameter	Experimental group				SD	<i>p</i> - value		
	Control	Ent M	Thymol	Ent M with Thymol		Ent M	Thymol	Interaction
AST (μ kat/L)	0.33	0.41	0.29	0.29	0.12	.4249	.1074	.3859
ALT (μ kat/L)	0.61 ^{ab}	0.88 ^a	0.78 ^a	0.44 ^b	0.27	.6286	.1116	.0009
GMT (μ kat/L)	0.07 ^a	0.12 ^a	0.09 ^a	0.08 ^a	0.04	.1503	.6642	.0264
ALP (μ kat/L)	1.87 ^a	2.71 ^a	2.66 ^a	2.27 ^a	0.60	.2457	.3713	.0037
Triglycerides (mmol/L)	1.51 ^{ab}	2.98 ^a	2.17 ^{ab}	1.32 ^b	1.15	.4065	.1822	.0038
Cholesterol (mmol/L)	1.06 ^{ab}	2.04 ^a	1.60 ^{ab}	0.87 ^b	0.77	.6068	.1890	.0011
Total protein (g/L)	53.84 ^{ac}	65.65 ^{ab}	68.24 ^b	50.38 ^c	10.43	.2635	.8708	<.0001
Urea (mmol/L)	4.64 ^a	6.28 ^a	6.02 ^a	5.73 ^a	1.51	.5190	.04040	.0014
Creatinine (μ mol/L)	75.33	76.33	95.14	83.50	23.15	.5340	.1230	.4608

^{a,b}Means within a row with different superscript letters differed significantly ($p < .05$). Data are presented as mean $\pm$ standard deviation (SD).

Ent M: enterocin M; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GMT, gamma glutamyl transferase; ALP: alkaline phosphatase.

Table 3. Effects of Enterocin M and thymol on antioxidant parameters and activity of lactate dehydrogenase in blood and tissues of rabbits ($n = 8$).

Parameter	Experimental group				SD	p-value		
	Control	Ent M	Thymol	Ent M with Thymol		Ent M	Thymol	Interaction
Blood								
GPx (μkat/g Hb)	2.20	2.42	1.54	3.13	1.53	.0966	.9610	.2038
MDA (nmol/mL)	0.33	0.35	0.31	0.34	0.03	.0548	.1730	.6087
Liver								
MDA (nmol/g protein)	87.21 ^a	65.85 ^b	62.87 ^b	78.57 ^{ab}	15.70	.5475	.2219	.0005
LDH (μkat/g protein)	16.76	17.50	17.89	19.24	2.77	.3020	.1600	.7611
Kidney								
MDA (nmol/g protein)	164.10 ^{ab}	230.00 ^a	148.20 ^b	215.20 ^a	51.36	.9696	.2925	<.0001
LDH (μkat/g protein)	16.02	16.04	16.87	19.01	2.83	.2641	.0536	.2729
Muscle								
MDA (nmol/g protein)	30.00	31.89	35.29	32.53	6.43	.8570	.2258	.3396
LDH (μkat/g protein)	23.89	23.73	24.44	22.24	6.34	.6512	.8568	.6958

^{a,b}Means within a row with different superscript letters differed significantly ($p < .05$). Data are presented as mean $\pm$ standard deviation (SD).

Ent M: enterocin M; GPx: glutathione peroxidase; MDA: malondialdehyde; LDH: lactate dehydrogenase.

and in plasma 47.38 ± 21.15 ng/mL (T) vs 42.81 ± 11.50 ng/mL (T + E, $p = 0.8506$). Thymol in muscle was under the limit of quantitation in both groups.

Biochemical parameters in serum

Application Ent M with thymol decreased ALT, triacylglycerides and cholesterol in comparison with separate addition ($p < .05$). Thymol addition alone increased urea concentration and total protein in serum ($p < .05$). There was no significant effect of thymol or Ent M application separately or together on AST, GMT, ALP and creatinine ($p > .05$, Table 2).

Antioxidant parameters and activity of lactate dehydrogenase in blood and tissues

Ent M ($p < .05$) and thymol ($p < .01$) separately significantly decreased MDA in liver. The activity of LDH showed a tendency to increase as a consequence of thymol addition either alone or with Ent M, in liver and kidney ($p > .05$). None of examined parameters in blood (GPx, MDA); kidney and muscle (MDA, LDH) were affected by the treatments ($p > .05$, Table 3).

Gut morphology

Ent M showed a tendency to increase the villus height-to-crypt depth ratio ($p = .0567$; none significant effect of both additives applicated together or separately was found ($p > .05$, Table 4).

Immunoglobulin A in jejunal wall and phagocytic activity in blood

Immunoglobulin A content in the intestinal wall was significantly higher during Ent M addition separately and in combination ($p < .05$). Thymol separately and in combination significantly increased phagocytic activity ($p < .05$). None significant effect of thymol separately application on IgA content ($p > .05$) and separately application of Ent M on phagocytic activity was found ($p > .05$, Table 4).

Concentration of organic acids in caecum

Lactic acid increased after thymol application alone and in combination ($p < .05$), Ent M did not affect its concentration ($P > .05$). Additionally, no effect on acetic, butyric and propionic acids concentration was

Table 4. Effects of Enterocin M and thymol on jejunal organ morphometric indices, and immunoglobulin A; and phagocytic activity in blood of rabbits ($n = 8$).

Parameter	Experimental group				SD	p-value		
	Control	Ent M	Thymol	Ent M with Thymol		Ent M	Thymol	Interaction
Vh (μm)	691.40	706.20	695.80	704.20	14.26	.0823	.8503	.6160
Cd (μm)	181.00	175.00	176.80	178.40	8.27	.5782	.9191	.3415
Vh/Cd	3.82	4.04	3.94	3.95	0.14	.0567	.9074	.0927
IgA ($\mu\text{g/g}$)	11.46	52.02	29.84	61.85	25.74	.0002	.0880	.5912
PA (%)	62.63	63.51	66.00	66.13	3.08	.6169	.0051	.7109

Data are presented as mean $\pm$ standard deviation (SD).

Ent M: enterocin M; Vh: villi height; Cd: crypt depth; Vh/Cd: villus height to crypt depth ratio; IgA: immunoglobulin A; PA: phagocytic activity.

Table 5. Effects of Enterocin M and thymol on organic acids concentration in caecum of rabbits ($n = 8$).

Parameter	Experimental group				SD	p-value		
	Control	Ent M	Thymol	Ent M with Thymol		Ent M	Thymol	Interaction
Acetic acid (mmol/L)	6.18 ^a	7.57 ^a	8.83 ^a	6.97 ^a	1.65	.7115	.1305	.0226
Lactic acid (g/100g)	16.64	15.78	19.92	22.16	4.46	.6994	.0142	.3903
Butyric acid (mmol/L)	1.56	1.82	2.19	1.56	0.60	.4941	.4918	.1063
Propionic acid (mmol/L)	0.43	0.63	0.57	0.46	0.18	.6083	.8497	.0576

Data are presented as mean $\pm$ standard deviation (SD).

Ent M: enterocin M.

detected during thymol and Ent M application separately or in combination ($p > .05$, Table 5).

Microbial evaluation

Thymol addition separately and in combination significantly decreased CoPS in the caecum ($p < .05$). Ent M separately and in combination decreased coliform bacteria in faeces ($p < .05$, Table 6). There was no effect of all three treatments on *Enterococcus* sp., LAB, CoNS in appendix, caecum and faeces ($p > .05$); on CoPS in appendix and faeces ($p > .05$); and on coliform bacteria in appendix and caecum ($p > .05$, Table 6).

Discussion

In the present experiment thymol in group with thymol application separately was in plasma 3-times and in group with combination thymol and Ent M 4-times lower than in jejunum, what again confirmed intensive absorption of thymol from intestinal tract and its passage through the intestinal wall as described in previous study Bacova et al. (2021). They found, that after 21 days application, thymol in plasma was 5.8-times lower than in jejunal wall as a consequence of constantly ongoing intensive biotransformation processes in enterocytes. We hypothesise that caecotrophy, the original feature of rabbit's digestion together with enteric and enterohepatic recycling of thymol are responsible for the higher amount of thymol in plasma during the longer application period (42 days).

Level of ALT in group with thymol and Ent M administration together significantly decreased in comparison with both separately applied additives (Table 2). According to Pogány Simonová et al. (2020a, 2020b), Ent M is able to regulate the balance of the gut microbiota and inhibit pathogenic bacteria and the release of toxic substances, which could avoid liver injury. ALT is aggregated primarily in the cytosol of hepatocytes and when reactive metabolites of oxidative stress initiate lipid peroxidation, the hepatocytes membrane integrity is impaired and enzyme is released into the blood. Bacova et al. (2020, 2021) pointed to intensive biotransformation processes of thymol in liver. Based on this finding we suppose that in our experiment, thymol after absorption from intestine passed through the portal vein to hepatocytes, where was metabolised and manifested its antioxidant properties and sparing effect on cell membranes. These two effects together, on gut microbiota (Ent M) and on hepatocytes (thymol) positively affected the liver function and subsequently decreased ALT level.

We found a similar effect on kidney biochemical parameters. Increasing levels of urea in the group with thymol addition again confirmed its intensive metabolism and accumulation in the kidney, as described by Ocelová (2017) and Bacova et al. (2020). Addition of both additives together slightly decreased the concentration of this parameter, probably as consequence of their synergistic effect, through modulation the intestinal microflora with Ent M, following improvement the thymol absorption from intestine and subsequently, its sparing effect on liver and renal function.

Table 6. Effect of Enterocin M and thymol on bacterial counts in appendix, caecum and faeces of rabbits (log 10 CFU/g, $n = 8$).

Parameter	Experimental group				SD	p-value		
	Control	Ent M	Thymol	Ent M with Thymol		Ent M	Thymol	Interaction
<i>Enterococcus</i> sp.								
appendix	1.68	2.40	1.99	3.00	1.46	.2153	.5033	.8353
caecum	1.27	0.90	0.90	1.53	0.60	.6303	.6303	.084
faeces	2.61	1.82	2.86	3.20	1.15	.6459	.1120	.2635
LAB								
appendix	2.60	2.55	2.62	3.09	1.03	.6840	.5741	.6014
caecum	3.02	3.66	2.71	3.20	0.72	.0844	.2252	.8060
faeces	3.66	3.54	3.41	3.62	0.35	.7302	.5161	.2205
CoNS								
appendix	2.45	2.05	2.48	1.99	0.72	.2001	.9671	.9061
caecum	3.24	3.51	3.04	3.60	0.74	.2391	.8784	.6680
faeces	3.25	3.14	3.27	3.03	0.35	.1818	.7212	.6067
CoPS								
appendix	2.70	3.21	4.29	2.88	1.30	.4283	.2653	.0998
caecum	2.75	2.89	2.14	2.31	0.61	.5433	.0329	.9445
faeces	3.23	3.13	3.10	3.78	0.63	.2992	.3402	.3402
Coliform bacteria								
appendix	1.18	1.83	3.15	2.12	1.43	.7629	.0887	.1954
caecum	2.19	3.51	3.17	3.69	1.37	.1455	.3497	.5156
faeces	5.73	2.62	4.96	3.57	1.57	.0002	.8450	.1798

Data are presented as mean $\pm$ standard deviation (SD).

Ent M: enterocin M; LAB: lactic acid bacteria; CoNS: coagulase-negative staphylococci; CoPS: coagulase-positive staphylococci; sp.: species.

It is necessary to underline that according with Washington and Van Hoosier (2012) value of ALT and urea (Leineweber et al. 2018) were in the normal range (ALT 0.23–1.3 μ kat/L, urea 2.63–10.28 mmol/L).

Rabbits obtain proteins directly from feed and caecotrophs they ingest, and their level in blood serum depends on absorption rate from intestine (Campbell-Ward 2012, Gasco et al. 2019). As was mentioned in our previous study (Bacova et al. 2020), thymol antioxidant properties improved the nutrient digestibility by a beneficial effect on intestinal wall structure. Pogány Simonová et al. (2020a, 2020b) showed that Ent M improved jejunal morphological parameters in rabbits. The gut-blood barrier serves as a protective barrier against entering pathogenic bacteria and toxins into the blood stream and consists of multiple layers, which protect intestinal villi from damage from luminal content, toxins and bacteria (Farhadi et al. 2003; Latek et al. 2022). We can hypothesise that if bacteriocins are able to fight with pathogenic bacteria and protect the intestinal wall against their adhesion, the structure of intestinal villi were improved and nutrients were better absorbed and utilised. Some tendency to improve the intestinal wall structure was found in present study after addition of Ent M together with thymol (Table 4).

Levels of triglycerides and cholesterol were in the normal range (0.83–8.89 mmol/L, 0.56–4.44 mmol/L; Washington and Van Hoosier 2012), but during application of Ent M and thymol together they had a decreasing tendency, which again confirmed the beneficial synergistic effect of both additives. As mentioned in Bacova et al. (2020), the antioxidant

properties of thymol possess a lipid-reducing function. We confirmed the inhibitory effect of thymol on MDA formation in the liver as a product of lipid peroxidation (Table 3). Bacteriocins are able to inhibit colonisation of the gastrointestinal tract with pathogens and prevent the crossing of toxins and other metabolites, thus reducing oxidative stress (Dicks et al. 2018, Pogány Simonová et al. 2020a, 2020b). The antioxidant effect of both additives prevented the formation of the investigated lipid parameters in liver.

We confirmed the beneficial effect of Enterocin M on the IgA concentration in intestinal wall, similarly as found by Pogány Simonová et al. (2020a, 2020b), who attributed the stimulation of secretory IgA production to improvement of the intestinal barrier and mucosal immune system through modulation of the intestinal microflora. As was mentioned by Placha et al. (2022b), due to its strong antioxidant properties, thymol is able to modulate phagocytic cells. We analysed a relatively high amount of thymol in plasma, and we assume that this concentration of thymol in blood circulation showed a sparing effect on phagocytic cells and consequently increased their activity.

The level of LDH showed an increased tendency in both groups with thymol addition, which again confirmed the intensive role of kidney in thymol biotransformation processes and probable creation of some substances, which could slightly alter cellular metabolic processes, as was mentioned in the studies of Ocelová (2017), Kohlert et al. (2002) and Bacova et al. (2020). These authors demonstrated that although the liver is the organ with intensive biotransformation processes, kidney microsomes are more effective in

metabolic processes then liver or intestinal microsomes. Thymol and its metabolites are able to be reabsorbed in the proximal tubule after glomerular filtration and its metabolites are cleaved by the activity of arylsulfatases to parental compound thymol, which is again reabsorbed. LDH is an intracellular enzyme and apoptosis of metabolic active cells may cause its increased release (Llana-Ruiz-Cabello et al. 2014). Phenolic compounds commonly have protective effect on cell structure, but only little is known about effect of its metabolites (Kohlert et al. 2002). We assume, that intensive biotransformation processes of thymol and its metabolites could be responsible for release of LDH from cells.

The antimicrobial activity of essential oils against Gram-positive and Gram-negative bacteria have been documented by several researchers (Franz et al. 2010, Gheisar and Kim 2018). Studies *in vitro* and *in vivo* demonstrated that the cell membrane of Gram-positive bacteria directly interact with lipophilic phenols, which disrupts their normal function (Yang et al. 2015). The amount of CoPS in our experiment was reduced only in caecum in the group with thymol addition. This was more than likely its concentration in caecum was higher as consequence of caecotrophy, which was confirmed in the previous study of Placha et al. (2022b). A reduction of coliform bacteria in faeces in groups with Ent M, may be attributed to the blocking of colonisation of intestinal wall by pathogenic microorganisms (Pogány Simonová et al. 2022). Although LAB did not increase in caecum, the higher concentration of lactic acids (Table 5) points to the stimulation activity of thymol on fermentation processes with subsequent changes in the microbial composition, in favour of a beneficial bacterial population. Cremonesi et al. (2022) also documented that Goji berry supplementation stimulated lactic acid fermentation, which caused changes in the intestinal microbiota composition.

Conclusions

The present study demonstrated the antibacterial effect of thymol against CoPS in caecum, the formation of MDA as product of lipid peroxidation in liver, and a stimulation effect on fermentation processes in caecum and on phagocytic activity in blood. Ent M decreased coliform bacteria in faeces and stimulated production of IgA in jejunum. Administration of Ent M together with thymol slightly improved the structure of jejunal wall and liver function. The obtained results showed the beneficial effect of thymol and Ent M

addition alone and also in combination. As their supplementation could reduce the incidence of health problems in animals, the obtained results should be helpful for farmers and in veterinary health care. This is a preliminary study on the effect of these two natural compounds, and to propose an appropriate dose, their bioavailability in the animal organism needs to be further studied.

Acknowledgements

The authors gratefully acknowledge the technical support provided by L. Ondruska, V. Parkanyi, R. Jurcik and J. Pecho from the National Agricultural and Food Centre, Research Institute for Animal Production, Nitra, Slovakia. The authors thank David McLean for improving the written English of the manuscript.

Ethical approval

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). The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The current experiment was financed with funds from the Scientific Grant Agency of the Ministry for Education, Science, Research and Sport of the Slovak Republic and the Slovak Academy of Sciences (Vega 2/0009/20) and was supported by the Austrian Federal Ministry for Science, Research and Economics, OeAD, Ernst-Mach Grant Action Austria-Slovakia (ICM-2019-13685) and Open-Mob-2022-01 and CNR-SAS-2022-02.

ORCID

Andrea Laukova  <http://orcid.org/0000-0001-7413-3816>
 Sona Gancarikova  <http://orcid.org/0000-0003-3813-8657>
 Margareta Takacsova  <http://orcid.org/0000-0002-7478-9177>
 Zuzana Andrejckova  <http://orcid.org/0000-0001-9929-0607>
 Monika Pogany Simonova  <http://orcid.org/0000-0002-0377-4672>
 Iveta Placha  <http://orcid.org/0000-0002-8724-0159>

Data availability statement

Data available on request from the authors.

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