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Quantitative PCR with 16S rRNA-gene-targeted specific primers for analysis of caecal microbial community in growing rabbits after dietary supplementation of thyme (*Thymus vulgaris*) and spirulina (*Arthrospira platensis*)

Viola Bagóné Vántus[a], Antonella Dalle Zotte[b], Marco Cullere[b], András Bónai[a], Alessandro Dal Bosco[c], Zsolt Szendrő[a], Gábor Tornyos[a], Roland Pósa[a], Brigitta Bóta[a], Melinda Kovács[a,d] and Attila Zsolnai[a]

[a]Agrár és Környezettudományi Kar, Élettani, Biokémiai és Állategészségügyi Intézet, University of Kaposvár, Kaposvár, Hungary; [b]Dipartimento di Medicina Veterinaria, Produzione e Salute, University of Padova, Legnaro, Italy; [c]Dipartimento Di Scienze Agrarie, Alimentari Ed Ambientali, University of Perugia, Perugia, Italy; [d]MTA-KE Mikotoxinok az Élelmiszerláncban Kutatócsoport, Kaposvár, Hungary

ABSTRACT

Objective of this study was to evaluate the effect of supplementation of growing rabbits' diet with 5% spirulina (*Arthrospira platensis*) and/or 3% (*Thymus vulgaris* L.), on composition and amount of rabbits caecal microbiota. After weaning, rabbits were randomly sorted to 4 groups ($n = 42$/group). The control group received a pellet without any supplementation, in treated groups the dietary supplementation was provided until 77 days of age. On 49, 63 and 77 days of age, six healthy animals/group were randomly selected and slaughtered. From the caecal content the amount of total bacteria, Bacteroides, *Clostridium leptum* and *Clostridium coccoides* were determined by quantitative polymerase chain reaction (qPCR), with the aid of bacterial ribosome coding DNA. The copy number of total bacteria, *C. leptum*, *C. coccoides* and Bacteroides varied between 2.75×10^{12} to 2.24×10^{13}, 5.25×10^{11} to 1.82×10^{12}, 2.5×10^{10} to 6.91×10^{11} and 5.89×10^{10} to 1.10×10^{12}, respectively. The amount of investigated bacteria represented 0.6%-13.4% of the total bacteria. The use of spirulina and/or thyme supplements influenced the amount of the copy number of each bacteria examined between 49 and 77 days of age. Their effect on the total bacteria number was temporary. Spirulina resulted in more Bacteroides on day 63 but significantly lower amount of Clostridia at 63 and at 77 days of age. The antimicrobial effect of thyme on the absolute Clostridia number was temporary, prevailing on day 63. Thyme resulted in significantly decreasing percentage ratio (within total bacteria) of *C. leptum* and *C. coccoides* by 77 days of age.

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Introduction

Microorganisms that live in coexistence with their hosts form the microbiota. Composition and roles of the bacteria that are part of this community have been intensively studied in the past few years. By far, the most heavily colonised organ is the gastrointestinal (GI) tract, and, as such, a large organ represents a major surface and rich in molecules that can be used as nutrients by microbes making it a preferred site for microbial colonisation (Sekirov et al. 2010). The GI mucosal surface is colonised by large numbers of heterogeneous bacteria that contribute to intestinal health and disease (DuPont and DuPont 2011). The rabbit digestive tract is adapted to process large amounts of fibre-rich feed by microbial fermentation which takes place in the caecum (Harcourt-Brown 2004). Composition and activity of caecal microbiota have a strong influence on health, because of their role in nutrition, pathogenesis and immune function (Gibson and Roberfroid 1995).

Rabbit production is currently concerned by digestive pathologies which are mostly encountered around weaning, leading to high morbidity and mortality (Bennegadi et al. 2003). Pre- and post-weaning periods are particularly important to establish resistance to digestive disorders in young rabbits and infectious

CONTACT Viola Bagóné Vántus violka36@gmail.com University of Kaposvár, Agricultural and Environmental Sciences, Guba S. 40, 7400 Kaposvár, Hungary

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digestive disorders account for a high incidence of mortality after weaning (Marlier et al. 2003). Although preventive medication with antibiotics (given under veterinary prescription) helps to control this period, there is an urgent need to find alternatives to maintain health with a reduced use of drugs (Gidenne et al. 2012). As alternative to antibiotics, several studies have been carried out on rabbits testing different feed additives (e.g. herbal extracts, pro- and prebiotics) because of their growth-promoting effects (Dalle Zotte et al. 2016).

Thyme has been attributed antimicrobial and antioxidant properties (Dorman and Deans 2000). According to Placha et al. (2013) a dietary supplementation of 5 g thyme essential oil/kg DM can improve intestinal integrity of rabbits, and it may have an antioxidant effect. Thyme essential oil increased *Lactobacillus* and decreased *E. coli* number in the ileum of Japanese quail (Khaksar et al. 2012), whereas it had no effect on the intestinal microflora populations in chickens from 7 days to 28 days of age (Cross et al. 2007). More recently, the addition of thyme leaves powder ameliorated the intestinal microflora through fortification of lactic acid bacteria in broiler chickens aged 14 and 28 days (Sadek et al. 2014).

Spirulina is a type of blue-green algae that is rich in protein, vitamins, minerals, and carotenoids. It has been used as human food supplement for over 20 years, because of its high nutrient content, including B complex vitamins, beta-carotene, vitamin E, manganese, zinc, copper, iron, selenium, and gamma linolenic acid (Belay et al. 1996). Several studies have been shown that Spirulina has beneficial biological activities, such as immunomodulation, antioxidant, anticancer, antimicrobial and probiotic effects (Belay 2002). In the rabbit, spirulina supplementation has been widely studied recently, and research gave exhaustive results on performance parameters and apparent digestibility of nutrients (Peiretti and Meineri 2008; Dalle Zotte et al. 2013; Gerencsér et al. 2014), meat quality and fatty acid composition of meat (Peiretti and Meineri 2011), carcase composition, vitamin B12 absorption into meat cuts, meat rheological traits and bones development (Dalle Zotte, Cullere, Sartori, Dal Bosco, et al. 2014), meat fatty acid profile and its oxidative status during retail display (Dal Bosco et al. 2014; Dalle Zotte, Cullere, Sartori, Szendrő, et al. 2014), serum biochemistry, immune response and antioxidant parameters (Kovács et al. 2016), oxidative stress (Kim et al. 2010) and atherosclerosis (Cheong et al. 2010), as well as on different effects in rabbits fed high fat diets (Meineri et al. 2009; Peiretti and Meineri 2009). Nevertheless, a limited number of studies explored the possible effect of blue-green algae on the bacteria colonising the GI tract, thus providing evidence that its dietary supplementation leads to alterations of the gut microbiota in mice Rasmussen et al. (2009).

The relationship between feedstuffs, feed supplements, microbial community and animal health, is still poorly understood (Michelland et al. 2011). Based on the complexity of the GI microbiota and the limited culturability of many of its members, it is clear that determining the function of all contributing microbes and their reaction on the applied treatment is a very difficult target. Since only 24%–40% of the microbial species of the microbiota can be cultured by classical microbiological methods (Tannock et al. 2000), molecular microbiology techniques are now used to provide more sensitive and accurate parameters for biodiversity and stability (Combes et al. 2013; Takahiro et al. 2003). Polymerase chain reaction (PCR) is a sensitive technique to detect sequences that are present at very low concentrations. More recently applied quantitative PCR (qPCR) method is the real-time PCR approach, which has been applied successfully to characterise GI content samples from various species. By real-time PCR bacterial targets can be quantified at a very low concentration, which is difficult or impossible using other (classical) approaches (Zoetendal et al. 2004).

In our previous paper, (Vántus et al. 2012) we discussed the effect of thyme and/or spirulina supplementation on the number of certain culturable bacteria and the fermentation processes in the caecum. In the current paper, we are focussing on changes in some of the important constituents of the caecal microbiota using molecular microbial approaches.

Materials and methods

Animals and experimental design

After weaning (35 days of age, 952 ± 81 g body weight) Pannon White rabbits were housed in wire-mesh cages (3 rabbits/cage; size of cage: 61 × 32 × 30 cm, length × width × height). The temperature and the photoperiod were 15–18 °C and 16L:8D, respectively. Before weaning (from 21 to 35 days of age) all rabbits received a pelleted control diet (C). After weaning rabbits were randomly sorted to 4 dietary groups ($n = 42$/group, i.e. 14 cages/group). The control group (C) received the C diet throughout the experiment (35–77 days of age, i.e. for 42 days). In treated groups, the C diet was supplemented with 5% spirulina (*Arthrospira platensis*) powder (S diet), or 3% thyme (*Thymus vulgaris* L.) powder (T diet) or both (ST diet) for the whole experimental period. All rabbits were fed ad libitum an isonitrogenous

(170–176 g/kg) and isoenergy (16.3−16.5 MJ gross energy/kg) basal diet. All diets had no anticoccidials or any other medication. Chemical composition of the supplements, ingredients and nutrients content of the experimental diets are summarised in Table 1 and 2.

At 49, 63 and 77 days of age (sampling times: 1, 2, 3), 6 healthy animals from each experimental group were randomly selected and slaughtered at 02:00 p.m. The digestive tract was removed and caecum was separated. Approximately 1.5 grams of fresh caecal content was placed into sterile Eppendorf tubes from each animal, thereafter frozen and stored at −80 °C until analyses for bacterial community.

Table 1. Chemical composition of Spirulina (*Arthrospira platensis*) and thyme (*Thymus vulgaris*) (g/kg).

Chemical composition	*Arthrospira platensis*	*Thymus vulgaris*
Dry matter	945	890
Crude protein	658	52.3
Crude fat	8.6	31.9
Crude fibre	nd[a]	182
Ash	65.1	65.9
Starch	35.6	58.4
Natural detergent fibre (NDF)	2.4	298
Acid detergent fibre (ADF)	4.8	210
Acid detergent lignin (ADL)	0.6	68.1
Ca	2.2	13.6
P	9.2	0.7
Ca/P	0.2	18.7
Gross energy (GE), MJ/kg	19.5	15.7

[a]not detected.

The research protocol was reviewed by the Animal Use and Care Administrative Advisory Committee and approved by the Agricultural Administrative Authority (Protocol No. 23.1/02322/006/2008).

DNA extraction and QPCR

Total DNA from about 200 mg of frozen caecal sample was extracted and purified using the QIAamp ® DNA Stool Mini Kit (50) (Qiagen, Duesseldorf, Germany) according to the manufacturer's instructions. DNA concentrations were measured using Smart Spec Plus Spectrophotometer (BioRad, Berkeley, California), based on absorbance at 260 nm.

After the preparation of the caecal samples (bacterial DNA extraction) the quantity of total bacteria, Bacteroides, *Clostridium leptum* and *Clostridium coccoides* were determined by qPCR reactions. Stratagene Mx 3000P QPCR apparatus (Agilent Technologies, Santa Clara, California) was used for bacterial target sequence amplification applying primers and SYBR Green in experimental assembly. The selection of the primers and the investigated bacterial groups was based on their role in digestion or in GI diseases; and on relevant scientific literature (Mariat et al. 2009; Angelakis and Raoult 2010; Xu et al. 2011), in which

Table 2. Ingredients and chemical composition of the experimental diets (g/kg).

	Diets			
	Control C	Spirulina	Thyme	Spirulina + thyme
Ingredients				
Spirulina	–	50	–	50
Thyme	–	–	30	30
Soybean meal 46%	130	55	140	60
Dehydrated alfalfa meal	400	397	370	397
Barley meal	247	262	237	262
Wheat straw (Faser-mix)	120	110	120	90
Dried apple pomace	40	40	40	40
Fat powder (40%)	35	35	35	35
Monocalcium phosphate	3	3	3	3
Salt (NaCl)	5	5	5	5
Methionine – DL	1	1	1	1
L-lysine HCL	4	6	4	6
Vitamin-mineral premix[a]	5	5	5	5
Zeolite	10	30	10	15
Analysed chemical composition				
Dry matter	896	898	898	896
Crude protein	176	170	175	172
Crude fat	25	26	27	28
Crude fibre	160	162	157	158
Starch	163	181	170	178
Ash	86	75	84	77
Natural detergent fibre (NDF)	323	316	314	301
Acid detergent fibre (ADF)	212	205	208	195
Acid detergent lignin (ADL)	53	45	53	46
Gross energy, MJ/kg	163	165	164	164

[a]Premix provided per kg of complete diet: vitamin A, 3.6 mg; vitamin D3, 25 μg; vitamin E acetate, 50 mg; vitamin K3, 2 mg; biotin, 0.1 mg; thiamine, 2 mg; riboflavin, 4mg; vitamin B6, 2 mg; vitamin B12, 0.1 mg; niacin, 40 mg; pantothenic acid, 12 mg; folic acid, 1mg; choline chloride, 300 mg; iron, 100 mg; copper, 20 mg; magnesium, 50 mg; cobalt, 2mg; iodine, 1mg; zinc, 100 mg; selenium, 0.1 mg.

Table 3. Oligonucleotide sequences used for QPCR.

Investigated group	Oligonucleotide sequence (5′–3′)	T_a (°C)[a]	References
Total bacteria			
Forward (27F_a)	AGA GTT TGA TYM TGG CTC AG	60	Fierer et al. (2008)
Reverse (338R_a)	GCT GCC TCC CGT AGG AGT		
Clostridium coccoides			
Forward (Cc1)	GAC GCC GCG TGA AGG A	60	Firmesse et al. (2008)
Reverse (Cc2)	AGC CCC AGC CTT TCA CAT C		
Clostridium leptum			
Forward (Cl8)	CCT TCC GTG CCG SAG TTA	60	Firmesse et al. (2008)
Reverse (Cl9)	GAA TTA AAC CAC ATA CTC CAC TGC TT		
Bacteroides			
Forward (Bs1)	CCT WCG ATG GAT AGG GGT T	60	Firmesse et al. (2008)
Reverse (Bs2)	CAC GCT ACT TGG CTG GTT CAG		

[a]T_a–Annealing temperature.

Table 4. Modification of total bacterial in the rabbit caecum according to three sampling times and four diets.

	Sampling point				
Diet	1 (49 days of age)	2 (63 days of age)	3 (77 days of age)	RSD	*p*-Value (sampling time)
C	$13.2 \pm 0.3^{a,A}$	$13.3 \pm 0.4^{a,B}$	13.6 ± 0.2^{B}	0.252	.008
S	$14.1 \pm 0.4^{b,A}$	$14.4 \pm 0.5^{b,B}$	13.5 ± 0.7^{A}	0.277	.001
T	$12.4 \pm 1.2^{a,A}$	$13.0 \pm 0.3^{a,A}$	13.8 ± 0.5^{B}	0.453	.000
ST	$13.1 \pm 0.7^{a,A}$	$13.3 \pm 0.8^{a,B}$	13.3 ± 0.5^{B}	437	.000
R squared	0.660	0.573	0.095		
p-Value (diet)	0.000	0.000	0.177		

Results are expressed as the mean of the log 10 value ± SEM calculated of targeted bacteria copy number in 1 gram of caecal sample.
Abbreviations mean – control (C), spirulina (S), thyme (T), spirulina and thyme (ST).
[a,b,c]Values within a column in the same sampling time, with different superscripts differ significantly at $p < .05$.
[A,B,C]Values within a row in the same diet, with different superscripts differ significantly at $p < .05$.

the same technical background was used for microbiota monitoring in different animal species.

QPCR was carried out in a 25 µL/tube reaction mixture containing 12.5 µL Brilliant II SYBR QPCR Low Rox Master Mix (Agilent Technologies, Santa Clara, California), 0.2 µM of each primer (Table 3), 10.5 µL sterile DEPC treated distillate water and 1 µL of DNA extract. Reaction mix manually compiled; and then, pipetted with QIAgility HEPA/UV workstation (for automated PCR setup, with UV light and HEPA filter) – 24 µL of reaction mix and 1 µL of sample in all tube. For PCR reactions 96-well plates were used. The robot was previously tested; an order of magnitude smaller standard deviation (SD) was founded, compared to SD of results obtained by manual setup.

The PCR programme consisted of 10 min at 95 °C, 40 cycles with 30 sec at 95 °C, 1 min at 60 °C. Specificity of PCR reactions was checked by melting point analysis. All samples were measured in technical triplicates. Ct values of the samples were the basis for monitoring the changes in the bacterial community. After cloning of the amplified PCR products (external lab orders), the plasmid concentrations were determined and dilution series to do standard curve were prepared. Conversion of plasmid concentration to copy number based on Lee et al. (2006). The bacterial content of the samples were calculated with the aid of standard curve derived from dilution series. The obtained copy numbers of the samples were adjusted to one gram of caecum.

Statistical analysis

The qPCR data set was analysed with the GLM (General Linear Model) procedure of SPSS programme (version 20), to determine differences between the amount of investigated bacterial groups, where the sampling points (1, 2 and 3), and diets (C, S, T and ST) were included as fixed effects and bacterial copy numbers as dependent variable. The formula of GLM included the following:

$y_{ij} = \mu + \text{sampling point}_i + \text{diet}_j + \text{sampling point}_i \times \text{diet}_j + e_{ij}$ where y is the copy number of the investigated bacteria (e.g. Bacteroides), µ is the general mean, sampling point is the event of caecal content sampling (1, 2, 3). Diet indicates the impact of the supplements (C, S, T and ST) and e is the residual error. The significance of differences was tested by least significant difference post hoc test.

Results

The amount of the studied bacterial groups can be seen in Tables 4–7 sorted by diet and sampling time.

Table 5. Modification of Bacteroides in rabbit caecal microbiota, according to three sampling times and four diets.

	Sampling point				
Diet	1 (49 days of age)	2 (63 days of age)	3 (77 days of age)	RSD	*p*-Value (Sampling time)
C	$11.2 \pm 0.3^{a,c,A}$	$11.3 \pm 0.5^{a,B}$	$11.7 \pm 0.3^{a,B}$	0.185	.035
S	$11.6 \pm 0.3^{a,A}$	$12.0 \pm 0.2^{b,B}$	$11.7 \pm 0.2^{a,A}$	0.364	.000
T	$10.8 \pm 0.1^{b,A}$	$11.1 \pm 0.6^{a,A}$	$12.0 \pm 0.3^{b,B}$	0.511	.000
ST	$11.2 \pm 0.2^{c,A}$	$11.4 \pm 0.2^{a,A}$	$12.0 \pm 0.6^{c,B}$	0.634	.000
R squared	0.371	0.476	0.401		
p-Value (diet)	0.000	0.000	0.000		

Results are expressed as the mean of the log 10 value ± SEM calculated of targeted bacteria copy number in 1 gram of caecal sample.
Abbreviations mean – control (C), spirulina (S), thyme (T), spirulina and thyme (ST).
^{a,b,c}Values within a column in the same sampling time, with different superscripts differ significantly at $p < .05$.
^{A,B,C}Values within a row in the same diet, with different superscripts differ significantly at $p < .05$.

Table 6. Modification of *Clostridium leptum* in rabbit caecal microbiota, according to three sampling times and four diets.

	Sampling point				
Diet	1 (49 days of age)	2 (63 days of age)	3 (77 days of age)	RSD	*p*-Value (Sampling time)
C	12.1 ± 0.3^{a}	12.1 ± 0.3^{a}	12.1 ± 0.3^{a}	0.010	.845
S	$12.1 \pm 0.2^{a,A}$	$11.9 \pm 0.3^{b,B}$	$11.8 \pm 0.2^{b,A}$	0.296	.001
T	$11.7 \pm 0.2^{b,A}$	$11.7 \pm 0.2^{b,A}$	$12.3 \pm 0.2^{a,B}$	0.605	.000
ST	$11.7 \pm 0.2^{b,A}$	$11.8 \pm 0.1^{b,A}$	$12.2 \pm 0.2^{a,B}$	0.509	.000
R squared	0.371	0.267	0.344		
p-Value (diet)	0.000	0.003	0.000		

Results are expressed as the mean of the log 10 value ± SEM calculated of targeted bacteria copy number in 1 gram of caecal sample.
Abbreviations mean – control (C), spirulina (S), thyme (T), spirulina and thyme (ST).
^{a,b,c}Values within a column in the same sampling time, with different superscripts differ significantly at $p < .05$.
^{A,B,C}Values within a row in the same diet, with different superscripts differ significantly at $p < .05$.

Clostridium leptum was found in the highest amount within total bacteria, followed by Bacteroides, whereas the amount of *Clostridium coccoides* was slightly less or equal to Bacteroides regardless of diet and sampling time.

At the 1st sampling time, that is, after 2 weeks of supplementation, the quantity of total bacteria was significantly higher in S group, compared to all other dietary groups (Table 4). The same was observed at sampling time 2 (i.e. on 63 days of age), but at sampling time 3 (slaughter age) no significant dietary effect was observed. This effect was not enforced when S was in combination with T supplement. Thyme by itself was not able to influence the amount of total bacteria either.

The quantity of Bacteroides was influenced by dietary supplementations, but changes were inconsistent (Table 5). In spirulina treated group, there was a temporary increase in their number, as can be seen on day 63 compared to control. On the other hand, treatment with thyme induced significant decrease after 2 weeks (by day 49), while by the next sampling time (day 63) no notable difference compared to control was found, and thereafter by day 77 more Bacteroides were present in the caecal content compared to C and S. Combined supplementation (ST) was only effective by the end of the experiment, and resulted more Bacteroides compared to C and S.

The two Firmicutes species examined (*Clostridium leptum* and *Clostridium coccoides*) showed very similar dietary response at first and second sampling time (Table 6–7). Thyme alone and in combination with S decreased Firmicutes number by day 49 and day 63 compared to C; whereas S exerted its antimicrobial effect by 63 days of age (2nd sampling time). The antimicrobial effect could be observed by the third sampling point in case of *C. coccoides*. Less (on average by 14%) *C. coccoides* was present in the caecum as compared to *C. leptum* during the whole experiment.

Regarding age, number of total bacteria slightly increased (C, T and ST) or did not change (S) during the experiment. Copy number of Bacteroides increased by age (i.e. sampling point), except S group, in which it remained in unchanged amount. Clostridia increased as time went by, except group S, in which there was a decrease by day 63.

Table 8 shows the ratio of investigated bacterial groups expressed as % of total bacteria in the rabbits' caecal chyme related to diet and sampling time.

Generally, the amount of investigated bacteria represented 0.6%-13.4% of the total bacteria, with the highest ratio (1%-7.4%) in the control, while the lowest (0.02-1.33) in the spirulina group. Bacteroides, *C. leptum* and *C. coccoides* were represented by 0.02%-4.26%, 0.33%–10.02% and 0.02%–1.42%, respectively.

Table 7. Modification of Clostridium coccides in rabbit caecal microbiota, according to three sampling times and four diets.

	Sampling point				
Diet	1 (49 days of age)	2 (63 days of age)	3 (77 days of age)	RSD	*p*-Value (Sampling time)
C	$11.3 \pm 0.3^{a,A}$	$11.5 \pm 0.4^{a,A}$	$11.8 \pm 0.4^{a,B}$	0.258	.007
S	$11.3 \pm 0.3^{a,A}$	$10.7 \pm 0.4^{b,B}$	$10.5 \pm 0.2^{b,B}$	0.593	.000
T	$10.4 \pm 0.2^{b,A}$	$10.6 \pm 0.3^{b,A}$	$11.1 \pm 0.2^{b,B}$	0.507	.000
ST	$10.4 \pm 0.2^{b,A}$	$10.6 \pm 0.1^{b,A}$	$11.0 \pm 0.2^{b,B}$	0.555	.000
R Squared	0.544 ,0	0.437	0.507		
p-Value (diet)	0.000	0.000	0.000		

Results are expressed as the mean of the log 10 value ± SEM calculated of targeted bacteria copy number in 1 gram of caecal sample.
Abbreviations mean – control (C), spirulina (S), thyme (T), spirulina and thyme (ST).
[a,b,c]Values within a column in the same sampling time, with different superscripts differ significantly at $p < .05$.
[A,B,C]Values within a row in the same diet, with different superscripts differ significantly at $p < .05$.

Table 8. Distribution of studied bacterial groups expressed as % of total bacteria related to diet and sampling time.

	Sampling point				
Diet	1 (49 days of age)	2 (63 days of age)	3 (77 days of age)	RSD	*p*-Value (sampling time)
Bacteroides					
C	1.54 ± 0.8	0.96 ± 0.4	1.23 ± 0.3	0.057	.368
S	0.21 ± 0.1	0.27 ± 0.1	0.72 ± 0.4	0.030	.647
T	1.11 ± 0.4^{a}	1.69 ± 1.1^{b}	2.80 ± 0.5^{c}	0.441	.000
ST	2.82 ± 0.8	3.60 ± 1.7	4.26 ± 1.7	0.214	.210
Clostridium leptum					
C	7.42 ± 3.0^{a}	$4.95 \pm 1.0^{a,b}$	$2,84 \pm 0.9^{b}$	0.215	.004
S	0.73 ± 0.2	0.33 ± 0.3	1.33 ± 0.8	0.079	.305
T	7.66 ± 1.1^{a}	6.58 ± 1.8^{b}	5.50 ± 1.3^{c}	0.466	.000
ST	10.02 ± 3.4^{a}	$8.14 \pm 1.8^{a,b}$	5.40 ± 1.2^{b}	0.263	.050
Clostridium coccoides					
C	1.09 ± 0.3	1.42 ± 0.6	1.11 ± 0.5	0.036	.536
S	0.15 ± 0.1	0.02 ± 0.0	0.06 ± 0.0	0.039	.559
T	0.46 ± 0.1^{a}	$0.51 \pm 0.1^{a,b}$	0.34 ± 0.1^{b}	0.181	.014
ST	0.51 ± 0.2	0.56 ± 0.1	0.29 ± 0.0	0.162	.317
Others					
C	89.94 ± 3.3^{a}	92.67 ± 2.0^{a}	94.81 ± 1.5^{b}	0.139	.025
S	98.92 ± 0.4^{a}	99.39 ± 0.3^{a}	97.89 ± 1.2^{b}	0.275	.004
T	90.77 ± 1.5^{a}	$91.22 \pm 1.4^{a,b}$	91.37 ± 1.7^{b}	0.264	.003
ST	86.64 ± 3.5^{a}	87.70 ± 1.9^{b}	$90.05 \pm 2.6^{a,b}$	0.220	.028

Abbreviations mean – control (C), spirulina (S), thyme (T), spirulina and thyme (ST).
[a,b,c]Values within a row in the same diet, with different superscripts differ significantly at $p < .05$.

Changes over time (age) can be observed in particular of *C. leptum* ratio, which reduced by one third (from 7.42 to 2.84%) in the control animals. In case of thyme, supplementation ratio of Firmicutes decreased. Spirulina supplementation increased the percentage ratio of *C. leptum* within total bacteria from (0,7% to 1,3%), but it was not significant. The combined application of thyme and spirulina resulted in decreased *C. leptum* ratio by the end of the experiment.

Discussion

In the rabbit, the bacterial community accounts for 10^{10}–10^{12}/g chyme in the caecum-colon tract (Forsythe and Parker 1985). Bacterial community cloning showed that the majority of the sequences (over 90%) were listed in the Firmicutes phylum, whereas Bacteroides, previously known as the predominant representative bacteria, were present only in 4% in an adult rabbit; Firmicutes groups established from the second week after birth and remained stable thereafter (Combes et al. 2011). The development of the rabbit caecal microbiota after birth has been thoroughly studied using 16S rRNA gene approaches coupled with capillary electrophoresis single-stranded conformation polymorphism (CE-SSCP) and qPCR by Combes et al. (2011). According to their results the total bacteria copy number increased with age, the Bacteroides-Prevotella copy number also increased from 14 to 21 days of age, whereas it decreased between 35 and 70 days of age. The copy number of Firmicutes did not change between 14 and 70 days of age. The amount of total bacteria and Bacteroides was less in their investigation when compared to our results, likely due to the different hybrids used, to rabbit nutrition and management, and also to different oligonucleotide sequences used for PCR. In our experiment, the copy number of total bacteria, *C. leptum*,

C. coccoides and Bacteroides varied between 2.75×10^{12} to 2.24×10^{13}, 5.25×10^{11} to 1.82×10^{12}, 2.5×10^{10} to 6.91×10^{11} and 5.89×10^{10} to 1.10×10^{12}, respectively. Age (sampling time) related changes in the copy number were influenced by the dietary treatments, that is, copy numbers increased except when spirulina was added to the diet.

The composition of the caecal microbiota in young rabbits is highly variable between individuals up to 49 days of age, whereas it becomes homogenous by 70 days of age (Combes et al. 2011). According to this statement after 49 days of age it is less possible to modify the microbiota, through nutritional factors.

In our experiment, the use of spirulina and/or thyme supplements influenced the amount of the copy number of the individual bacteria examined between 49 and 77 days of age. On the other hand, their effect on the total bacteria number was only temporary (see spirulina supplementation on day 63) and no difference attributed to the supplementation compared to the control animals was detected on day 77. There were more bacteria (total bacteria number) at 77 compared to 49 days of age, except in group S, where no increase was observed.

Several pro- and prebiotics have been described to modify the composition of the gut microbiota, however the modulation appears to be restricted usually only to certain bacterial groups (Macfarlane et al. 2008). Spirulina is less known as a pro- or prebiotic feed supplement. According to Rasmussen et al. (2009) feeding 5% Spirulina platensis induced changes in the murine gut microbiota, but the microbial community remained around 70% similar to that of the control animals. In our experiment, dietary inclusion of spirulina resulted in more Bacteroides on day 63 but significantly lower amount of Clostridia at 63 and at 77 days of age. Investigating the ratio of the examined bacterial groups within total bacteria, we can conclude that spirulina increased the ratio of the sum of Bacteroides, *C. leptum* and *C. coccoides* as time went by, while the other three treatments led to the increase of the ration of other, not identified bacteria. The mechanism by which spirulina modulates microbiota composition, and by which directly or indirectly decreased the ratio of these bacteria is not known yet, it is suggested to be due to selective antimicrobial activity, or it may serve as substrate for certain microbes (Rasmussen et al. 2009).

The antimicrobial effect of thyme on the absolute Clostridia number was only temporary, prevailing on day 63. On the other hand, thyme resulted in significantly decreasing percentage ratio (within total bacteria) of *C. leptum* and *C. coccoides* by 77 days of age. Results support previous data according to which thyme (due to its volatile oils) exerts antimicrobial effects. Dorman and Deans (2000) demonstrated that among many volatile oils tested, the oil (thymol) of *Thymus vulgaris* was found to have the widest antibacterial spectrum. Thymol has a phenolic structure and exerted greater inhibitory activity against gram-positive bacteria, Clostridia included.

Conclusions

In this study, spirulina and thyme included separately or combined in rabbit diets affected the composition of the caecal microbiota in weaned rabbits between 35 and 77 days of age. They affected the absolute number of total bacteria, Bacteroides, *C. leptum* and *C. coccoides*, and also the ratio of the mentioned bacteria within the total bacterial community. Spirulina dietary supplementation temporary increased the amount of total bacteria and Bacteroides, while resulted in less Clostridia. Thyme exerted its antimicrobial effect on Clostridia only till 63 days of age. In this study, in the potential use of these additives to modify the composition of the microbiota has been demonstrated, but further research is advisable to optimise effects on rabbits' microbial balance before practical suggestions can be recommended.

Disclosure statement

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ORCID

Viola Bagóné Vántus http://orcid.org/0000-0002-4021-2281
Antonella Dalle Zotte http://orcid.org/0000-0002-4021-2281
Marco Cullere http://orcid.org/0000-0002-4021-2281
András Bónai http://orcid.org/0000-0002-4021-2281
Alessandro Dal Bosco http://orcid.org/0000-0002-4021-2281
Zsolt Szendrő http://orcid.org/0000-0002-4021-2281
Melinda Kovács http://orcid.org/0000-0002-4021-2281

Attila Zsolnai http://orcid.org/0000-0002-4021-2281

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