DIETARY SUPPLEMENTATION OF SPIRULINA (*Arthrospira platensis*) AND THYME (*Thymus vulgaris*). PART 2: EFFECT ON GASTROINTESTINAL GROWTH, CAECAL MICROBIOTA AND FERMENTATION IN RABBITS

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ABSTRACT

The objective of this study was to evaluate the effect of a dietary supplementation with Spirulina or/and Thyme on the rabbit gastrointestinal tract development (from 5 to 11 weeks old), the composition of the microbiota and production of volatile fatty acids. Four groups of 21 rabbits weaned at 35 days of age and then fed ad libitum, either a control diet or the same diet supplemented with 5% Spirulina or 3% Thyme, or both. Six healthy animals from each group were randomly selected and sacrificed at 35, 56 and 77 days of age. No significant differences were detected in body weight (bw) between the groups. Relative weight of kidneys, stomach, small intestine, caecum decreased with age from 0.8, 1.5, 3.0, 1.5% bw to 0.6, 1.1, 2.5, 1.4% bw resp. No diet effect on pH of the small intestine and the caecal content was detected, while gastric pH increased with age from 1.5 to 2.0. Number of *E. coli*, total anaerobic and strictly anaerobic bacteria decreased with age (from $\log_{10}$ 3.9 to 3.2, 8.8 to 8.4 and 8.1 to 7.8 resp.). No effect of the diet could be demonstrated. Supplementation with Thyme has no significant effect on caecal VFA profile. In conclusion, Spirulina and/or Thyme supplementation of diet after weaning had no substantial effect on the growth of the gastrointestinal tract, the composition of the caecal microbiota and VFA production.

Key words: Spirulina, Thyme, gastrointestinal growth, caecal fermentation, caecal microbiota, Rabbit

INTRODUCTION

Spirulina is a type of blue-green algae that is rich in protein, vitamins, minerals, and carotenoids. It contains nutrients, including B complex vitamins, beta-carotene, vitamin E, manganese, zinc, copper, iron, selenium, and gamma linolenic acid (Belay *et al*., 1986). It has been used as human food supplement for over 20 years, but its use as an animal feed supplement has become more current in the last few years. According to Peiretti and Meineri (2008) 15 % *Spirulina platensis* in the fattening period, can be used in rabbit diets, without any adverse effects on growth performance. Though Spirulina containing diet had lower digestibility, this was balanced by an increased feed consumption.
In the last decades, herbal extract feed additives have also been widely used as alternatives to antibiotics because of their growth-promoting effects. Thyme has been reported for its antimicrobial and antioxidant properties (Dorman and Deans, 2000). The dietary application of phytobiotic preparation, containing also Thyme, caused a decrease in small intestinal viscosity, lower weight of caecal and colonic contents, diminished activity of microbial enzymes, and limited production of short chain fatty acids in the caecum of rabbits. The improved growth performance was attributable to the antimicrobial effect of the herbs (Zduncziyk *et al*., 2011). The use *Thymus vulgaris* did not cause any adverse effect on histological structure of liver, kidney and testis in rabbits (Tousson *et al*., 2011).

Our experiment belongs to a larger study on the effect of Spirulina and Thyme dietary supplementation for the growing rabbit (Gerencsér *et al*., 2012; Vantus *et al*., 2012). The objective of this trial was to evaluate the effect of supplementation (between the ages of 5-11 weeks) of the growing rabbits' diet by Spirulina (5%) or/and Thyme (3%) on the growth of the gastrointestinal tract, the composition of the microbiota and production of volatile fatty acids.

MATERIALS AND METHODS

Experimental animals and housing

Experiment was established with Pannon White rabbits in experimental farm of Kaposvar University. Young rabbits were weaned at 35th day of life. Weaned rabbits were randomly sorted into four groups according to diet. Every group contained 21 weaned rabbits, housed by three in wire net cage. Water and pelleted diet were available *ad libitum*. Average temperature ranged 16 to 18°C, lighting cycle was 16 hours light: 8 hours dark (time=22:00-6:00), and the farm had overpressure ventilation.
The research protocol was reviewed by the Animal Use and Care Administrative Advisory Committee and approved by the Agricultural Administrative Authority (Protocol No. 00618/007/SOM/2003).

Experimental diets

Pellet of control group was fed to does and kits from 21st day of life. Weaned rabbits were allocated into four groups. The control diet (C) was formulated with no supplementation, while the others were supplemented with 5% Spirulina (*Arthrospira platensis*) (S) and 3% Thyme (*Thymus vulgaris L.*) (T), or both (ST), respectively, throughout the experiment (5-11 weeks of age). More details about housing and feeding conditions are described in Gerencsér *et al.* (2012).

Sample collection

Six healthy animals from each group were randomly selected at 35, 56 and 77 days of age. Rabbits were sacrificed at 13:00. The digestive tract was removed and the stomach, the small intestine and the caecum were separated. The quantity of the fresh gastric, small intestinal and caecal contents was measured and their pH values were determined. Weight of the liver, heart, kidneys, lung as well as of the empty stomach, small intestine and caecum was measured, relative weights were calculated (i.e. weight of the organ expressed in % of body weight).

Microbiological determination

The fresh caecal content was homogenised at room temperature. Serial dilution was made from this sample with sterile isotonic solution. Total aerobic bacteria and total anaerobic bacteria were cultured on bloody agar, prepared with 5% defibrinated calf blood. Samples were incubated at 37^{o}C for 24 h and 72 h, under aerobic and anaerobic condition, respectively. Coliforms and *Escherichia coli sp.* were cultured on a Chromocult differentiation medium (Merck, Darmstadt, Germany). Samples were incubated at 37^{o}C, under aerobic conditions, for 24 h. *Campylobacter* sp. was cultured on blood-free selective agar (Merck). Samples were incubated at 37^{o}C, under aerobic conditions, for 48 h. The obligate anaerobe organisms were cultured on Schaedler's agar (Scharlan Chemie, Barcelona, Spain), the selectivity of which was increased by the addition of esculin, neomycin (Merck) and Ferri-ammonium-citrate (Scharlan Chemie, Barcelona, Spain). Petri dishes were placed into Anaerocult culture dishes (Merck), in which the anaerobic conditions were ensured with the help of an Anaerocult A (Merck) gasifying bag. The samples were incubated 37^{o}C for 96h.
After the incubation had elapsed, the colonies were counted (ISO 4833:2003) with Acolyte colony counter (Aqua-Terra Lab, Veszprem, Hungary). The colony counts were expressed in $\log_{10}$ colony forming units (CFU) related to 1g of sample.

Determination of volatile fatty acids (VFA) concentration

VFA concentrations of 11 week old growing rabbits' caecal samples were measured. Approximately 5g digesta per rabbit were stored in fridge (-20 ^{o}C) until measurement. The concentration of VFA was measured by gas chromathography (Shimadzu GC 2010, Japan), using 2-etil-butyrate (Fluka Chemie GmbH, Buchs, Switzerland) as internal standard.

Statistical Analysis

Data were analysed by using GLM procedure of SPSS (2002), version 10.0. Group of diet and age were factors. VFA content of 11th week old rabbits' caecal digesta was analysed by using one way ANOVA. The significance of differences was tested by Tukey post hoc test.

RESULTS AND DISCUSSION

Table 1: Effect of Spirulina or Thyme supplementation on digestive organ development* of the growing rabbit

	Experimental diets				Age (d)			*RSD*	*P-value*		
	Control	Spirulina	Thyme	Spi+Thy	35	56	77		*Diet*	*Age*	*D*A*
n	18	18	18	18	24	24	24				
Body weight (g)	2069	2032	2074	2069	1615^{a}	2035^{b}	2533^{c}	*401*	*0.79*	*<.001*	*0.57*
Heart %*	0.3	0.3	0.3	0.3	0.3^{b}	0.3^{a}	0.3^{a}	*0.0*	*0.45*	*0.00*	*0.45*
Liver %*	3.7	3.6	3.5	3.9	3.7	3.8	3.5	*0.5*	*0.08*	*0.12*	*0.59*
Kidneys %*	0.8	0.7	0.7	0.7	0.8^{c}	0.7^{b}	0.6^{a}	*0.1*	*0.13*	*0.00*	*0.14*
Spleen %*	0.1	0.1	0.1	0.1	0.1	0.1	0.1	*0.0*	*0.63*	*0.16*	*0.05*
Stomach %*	1.3	1.3	1.3	1.3	1.5^{c}	1.3^{b}	1.1^{a}	*0.2*	*0.33*	*0.00*	*0.52*
Small intestine %*	2.8	2.7	2.7	2.7	3.0^{c}	2.7^{b}	2.5^{a}	*7.6*	*0.24*	*0.00*	*0.70*
Caecum %*	1.5	1.5	1.5	1.4	1.5^{b}	1.5^{ab}	1.4^{a}	*0.2*	*0.18*	*0.03*	*0.08*
Appendix %*	0.6	0.5	0.5	0.5	0.5	0.5	0.6	*0.1*	*0.17*	*0.51*	*0.43*

RSD: residual standard deviation; means from 6 rabbits per age and diet.
a, b: means wearing the same superscript did not differ between ages, at the level P=0.05.
* : relative weight of the organs expressed in % of body weight

No significant differences in body weight or the relative weight of the organs were observed due to supplementation the diet with Spirulina, Thyme or both (Table 1). This is in accordance with the finding of Peiretti and Meineri (2008), whose result showed, that increasing level (5%, 10% and 15%) of Spirulina did not influence final weight, weight gain and feed efficiency in rabbits. In hypercholesterolemia induced rabbits 1 or 5 % Spirulina fed for 8 weeks did not affect the weight of the spleen, kidney and heart and the increase of liver weight was not attenuated by Spirulina treatment (Cheong *et al*., 2010).

Table 2: Effect of Spirulina or Thyme supplementation on digesta traits and caecal microbiota of growing rabbits

	Experimental diets				Age (d)				*P-value*		
	Control	Spirulina	Thyme	Spi+Thy	35	56	77				
n	*18*	*18*	*18*	*18*	*24*	*24*	*24*	*RSD*	*Diet*	*Age*	*D*A*
pH of stomach content	1.69	1.88	1.76	1.66	1.53^{a}	1.67^{ab}	2.03^{b}	*0.62*	*0.80*	*0.021*	*0.77*
pH of small intestine. content	7.63	7.67	7.66	7.63	7.62	7.63	8.00	*0.18*	*0.83*	*0.24*	*0.25*
pH of caecal content	6.37	6.46	6.44	6.49	6.35	6.50	6.47	*0.23*	*0.50*	*0.12*	*0.83*
Total aerobic bacteria [1]	6.91	6.87	7.02	6.53	7.02	6.91	6.57	*0.71*	*0.13*	*0.055*	*0.11*
Total anaerobic bacteria [1]	8.61	8.52	8.54	8.39	8.82^{b}	8.31^{a}	8.39^{a}	*0.43*	*0.36*	*<0.001*	*0.093*
Strictly anaerobic bacteria [1]	7.95	7.86	8.00	7.92	8.05^{b}	8.00^{b}	7.75^{a}	*0.35*	*0.64*	*0.005*	*0.46*
E. coli sp. [1]	3.95	3.92	3.70	3.50	3.91^{b}	4.19^{b}	3.21^{a}	*0.84*	*0.22*	*<0.001*	*0.34*
Campylobacter sp. [1]	3.81	4.01	3.59	3.51	3.80	3.66	3.01	*0.86*	*0.54*	*0.58*	*0.93*

RSD: residual standard deviation; means from 6 rabbits per age and diet. [1]Germ counts expressed in $\log_{10}$ CFU/g caecal digesta. Different superscripts mean significant differences between ages.

Relative weight of the heart, the kidneys, the stomach, the small intestine and the caecum decreased with age. According to our previous results the experimental animals age was between 14 and 42 days. In that experiment the relative weight of the liver and gastrointestinal tract increased from 2.9% and 5.5% to 3.7% and 10.3%, respectively. At the other hand, the relative weight of heart, kidneys and lung decreased from 2.8% to 1.9% (Kovács *et al*., 2011).

No diet effect on pH of the small intestine and the caecal content was detected (Table 2), while gastric pH increased with age from 1.5 to 2.0. Number of *E. coli*, total anaerobic and strictly anaerobic bacteria decreased by age, no effect of the diet could be demonstrated. Supplementation with Thyme did not affect the caecal VFA profile (Table 3).

Detecting no change in the number of the cultured bacteria is frequently experienced when examining effects of different diets. Only 24 to 40 % of the microbial species of the microbiota can be cultured in vitro, so molecular microbiology techniques are recently used to provide more sensitive and accurate parameters for biodiversity and stability. These results were published in Vántus *et al*. (2012).

Table 3: Effect of Spirulina or Thyme supplementation on VFA content of the caecal digesta of 77 days old rabbits*.

Traits	Experimental diets								*P-*
n = 6	Control*	*sd*	Spirulina	*sd*	Thyme	*sd*	Spi+Thy	*sd*	*value*
Dry matter content (w/w%)	22.6	*1.1*	22.5	*1.6*	22.6	*0.4*	22.5	*0.9*	*0.99*
Total VFA content (mmol/kg dry matter content)	35.1	*5.4*	31.0	*9.6*	34.3	*6.4*	36.1	*10.0*	*0.72*
Acetic acid %[1]	74.8	*1.8*	73.6	*1.60*	74.1	*1.12*	73.9	*1.2*	*0.52*
Propionic acid %[1]	7.0	*0.5*	7.2	*1.3*	8.5	*1.3*	7.8	*0.9*	*0.10*
Butyric acid %[1]	18.2	*1.8*	19.3	*2.2*	18.8	*4.3*	17.3	*2.1*	*0.66*

[1] Proportion within total VFA content

*means and standard deviation (sd) from 6 rabbits per diet.

CONCLUSIONS

Supplementing the diet by Spirulina and/or Thyme after weaning had no substantial effect on the growth of the gastrointestinal tract, the composition of the caecal microbiota and VFA production. This may be presumably due to the relatively late beginning of the supplementation, i.e. pro- and prebiotic like substances should be applied already before weaning to have distinct effects on digestive physiological parameters.

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