

**DIETARY SUPPLEMENTATION OF SPIRULINA (*Arthrospira platensis*) AND THYME (*Thymus vulgaris* L.).
PART 3: EFFECT ON CAECAL BACTERIAL COMMUNITY IN GROWING RABBITS**

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ABSTRACT

Two groups of 42 rabbits were fed either a diet supplemented or not with Spirulina Duration, from weaned (35d old) to 11 weeks of age. Samples of caecal content were collected at the 14th, 28th and 48th day of the supplementation, to evaluate the effect on the bacterial diversity of microbiota using molecular tools. After bacterial DNA extraction, the quantity of bacteria, belonging to phylum *Firmicutes* and *Bacteroidetes* were determined by qPCR reactions. The copy number of the two phyla did not significantly evolved from 49 to 83d of age, and ranged between 87000 and 118000. The amount of Bacteroidetes and Clostridium group (*Clostridium leptum* and *Clostridium coccooides*), belonging to the *Firmicutes* phylum, were not significantly modified by Spirulina dietary supplementation.

Key words: Spirulina, caecal microbiota, rabbit, qPCR

INTRODUCTION

Post-weaning mortality in rabbit production is primarily due to diseases of the digestive tract (Gidenne and Fortun-Lamothe, 2002). The composition and the activity of the caecal microbiota could have strong influence on health, because of its role in nutrition, pathogenesis and immune function (Gibson and Roberfroid, 1995). Since only 24 to 40 % of the microbial species of the microbiota can be cultured in vitro (Tannock *et al.*, 2000), molecular microbiology techniques are now used to provide more sensitive and accurate parameters for biodiversity and stability (Takahiro *et al.*, 2003; Combes *et al.*, 2012).

In connection with the ban of using antibiotics as growth promoters in the EU several studies have been carried out on different feed additives as alternatives for antibiotics. Spirulina 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.*, 1986). Several studies have been shown that Spirulina has several biological activities, such as immunomodulation, antioxidant, anticancer, antimicrobial and probiotic effects (Belay, 2002).

Our experiment belongs to a larger study on the effect of Spirulina and Thyme dietary supplementation for the growing rabbit (Gerencsér *et al.*, 2012; Bonai *et al.*, 2012). We here aimed to evaluate the effect of a 5% Spirulina supplementation on the bacterial diversity of the rabbit caecal microbiota.

MATERIALS AND METHODS

Animals and experimental design

The experiment was conducted at the experimental rabbit farm of Kaposvár University. All rabbits received the control pellet (C) from the age of 3 weeks. After weaning (35d old) the rabbits were housed in wire net cages (0.61 x 0.32m, 3 rabbits/cage). The temperature in the house was 15-18°C

and daily lighting was 16 hours. The weaned rabbits were randomly sorted to 2 groups (42 rabbits/group). Rabbits of the control group (C) received a pellet without any supplementation throughout the experiment (35 to 77d old). In the treated group, the pellet was completed by 5% Spirulina (S), for the whole growth period (35-77d) growing period. More details about housing and feeding conditions were described by Gerencsér *et al.* (2012).

At 14, 28 and 42 days after weaning (i.e. 49, 63 and 77d old resp., for sampling points: 1, 2, 3, resp.), 6 healthy animals from each group (one animal/cage) were randomly selected and slaughtered at 02:00 pm. The digestive tract was removed immediately and the caecum was separated. The quantity of the fresh caecal contents was measured thereafter frozen and stored at -80°C until analysed for bacterial community changes.

DNA extraction and QPCR

Total DNA from about 200 mg of caecal sample was extracted and purified using the QIAamp® DNA Stool Mini Kit (50) (QIAGEN) according to the manufacturer's instructions. DNA concentrations were measured using Smart Spec Plus Spectrophotometer (BioRad). The concentrations of all DNA samples were set to 60 ng/ μl .

After the preparation of caecal samples (bacterial DNA extraction) the quantity of bacteria (belonging to phylum Firmicutes and Bacteroidetes) were determined by qPCR reactions. The primer and investigated bacterial group selection were based on those scientific literature (Mariat *et al.*, 2009; Angelakis and Raoult, 2010; Xu *et al.* 2011) where microbiota monitoring has been performed in different species on the same technical background. 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), 0.2 μM of each primer (Table 1.), 10.5 μl sterile distilled water and 1 μl of DNA extract (60 ng/ μl). The PCR program consisted of 10 min at 95°C , 40 cycles with 30 sec at 95°C , 1 min at 60°C .

Table 1. Oligonucleotide sequences used for QPCR.

Group	Item	Oligonucleotide sequence (5'-3')	T _a *($^{\circ}\text{C}$)	References
<i>Clostridium</i> <i>coccoides</i>	Forward (Cc1)	GAC GCC GCG TGA AGG A	60	Firmesse <i>et al.</i> (2008)
	Reverse (Cc2)	AGC CCC AGC CTT TCA CAT C		
<i>Clostridium</i> <i>leptum</i>	Forward (Cl9)	CCT TCC GTG CCG SAG TTA	60	Firmesse <i>et al.</i> (2008)
	Reverse (Cl8)	GAA TTA AAC CAC ATA CTC CAC TGC TT		
<i>Bacteroides</i>	Forward (Bs2)	CCT WCG ATG GAT AGG GGT T	60	Firmesse <i>et al.</i> (2008)
	Reverse (Bs1)	CAC GCT ACT TGG CTG GTT CAG		

*T_a – Annealing temperature

MxPro 3000P QPCR apparatus (Agilent Technologies) was used for the bacterial target sequence amplification applying primers and SYBR Green in the experimental assembly. Specificity of PCR reactions were checked by melting point analysis. All samples were measured in three technical triplicates. C_t values of the samples -having equilibrated concentrations- were the basis of monitoring the changes of bacterial community. After cloning of the amplified PCR products (external Lab. orders), we determined the plasmid concentrations, and dilution series were prepared (standard curve). The bacterial contents of samples were calculated with the aid of that. The obtained copy numbers of the samples were adjusted to one gram of caecum.

Data analysis

The copy numbers of the samples were visualised in copy numbers vs. sampling-events coordinates (data not shown). Deviations of the copy numbers have been calculated. Testing for significant differences between the sampling points (in case of Spirulina supplement and in case of the control group separately) has been accomplished by t-test.

RESULTS AND DISCUSSION

All investigated bacterial group showed no significant changes in amount according to during the experimental period. Similarly, no significant changes were detected between the control and the Spirulina group, whatever the bacterial groups *Bacteroides* or *Clostridium* (Table 2.).

Table 2. Average* copy number of 1 gram caecum samples, according to age and Spirulina supplementation in the diet of the growing rabbit

Sampling point (day of the treatment)	Number of copies											
	Bacteroides				Clostridium leptum				Clostridium coccoides			
	Control	Deviation	Spirulina diet	Deviation	Control	Deviation	Spirulina diet	Deviation	Control	Deviation	Spirulina diet	Deviation
1 (14)	94842	6220	91839	2969	93739	5975	90402	2888	106955	6905	103154	3214
2 (28)	97091	8165	98657	4347	95784	7879	96411	4399	109410	9143	110365	5227
3 (48)	98272	6126	96539	7901	96671	6444	94550	7846	110086	7684	107853	9105

*Means for 6 replicates per group and per age: No significant differences were detected among sampling points or diets, for any bacterial group.

Sampling point 1, 2 and 3 were 49, 63 and 77d old resp.

In the same way, any significant differences according to age were observed for the ratio of different bacterial groups (Table 3).

Table 3. Copy number ratio* according to age of the growing rabbit

Sampling point	Copy numbers relative to another		
	Bs / CI	BS / Cc	CI / Cc
1 (day 14)	1.02	0.89	0.88
2 (day 28)	1.02	0.89	0.87
3 (day 48)	1.02	0.90	0.88

Bs – *Bacteroides* - Cc - *Clostridium coccoides* - CI - *Clostridium leptum*

*Means for 12 replicates per age: No significant differences were detected among sampling points for any bacterial group.

The observed trends -in case of Firmicutes- are in good accordance of the findings in humans and rabbit, such as body weight (Turnbaugh *et al.*, 2009) and age (Combes *et al.*, 2011) are associated with a larger proportion of Firmicutes.

CONCLUSIONS

Since we were not able to notice significant change in some microorganisms of the caecal microbiota according to Spirulina; we can conclude, that a 5% Spirulina supplementation would not affect the main phyla of the caecal microbiota in the growing rabbit.

The robustness of qPCR and the chance to notice significant changes of microbiota can be improved by decreasing the variance via automated sample handlings. Further insight of the changes can also be achieved by monitoring total bacterial copy number and/or other type of bacteria e.g.: bifidobacteria.

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