THESIS OF DOCTORATE (Ph.D.)
DISSERTATION

UNIVERSITY OF KAPOSVÁR

FACULTY OF AGRICULTURAL AND ENVIRONMENTAL SCIENCES

Institute of Physiology, Biochemistry and Animal Health

Head of Doctorate School:
Dr. Kovács Melinda
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EXAMINATION OF THE EFFECTS OF CERTAIN FACTORS INFLUENCING CAECAL FERMENTATION IN RABBITS

Written by:

BÓNAI ANDRÁS

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1. BACKGROUND OF RESEARCH, OBJECTIVES

Young rabbits are highly sensitive to multifactorial diseases of the digestive system. These diseases are often severe and may be fatal. About one-fourth of all mortality takes place in the period around weaning. High morbidity and mortality have serious economic impact on meat production and negative effect on animal welfare.

Antibiotics were widely used to reduce mortality of the growing rabbit. Due to the restriction of antibiotic use in animal production, a decrease of the previously established yields has to be reckoned with, which has major economic implications. Rabbit producers have to face smaller profits due to the lower production yields, and researchers have to find new solutions suitable for replacing the use of antibiotics.

Several studies have been carried out on different feed additives as alternatives to antibiotics. Probiotics are viable microorganisms that promote the beneficial balance of the microbial population in the gastrointestinal tract. The prebiotic concept is based on the assumption that certain beneficial gut bacteria may be selectively stimulated by undigestible but fermentable dietary carbohydrates. These alternative feed additives have been intensively studied in livestock, but their effect is often unconsistent.

Early weaning age is a commonly used method in rabbit breeding and it could be influence the development of digestive system and the microbiota in rabbit caecum.

I studied the operation of gastrointestinal tract around weaning in domestic rabbits, and looked for solutions suitable for replacing the use of antibiotics during the growing period in rabbits. I tried to determine the weaning age that is optimal from the microbiological point of view, and conducted experiments to determine the effects of probiotic and prebiotic added to the diet.

The aims of the present work were the followings:

1.) Determination of the effect of different weaning ages on the growth and certain parameters of digestion in rabbits.

2.) Determination of the effect caused by the probiotic *Bacillus cereus* var. *toyoi* and by inulin, a compound having prebiotic effect, on the composition of the caecal microbiota and on caecal fermentation activity in the period around weaning in rabbits.

3.) Elaboration of a series of objective and complex biomonitoring methods to characterise the caecal microbiota and caecal fermentation processes of rabbits, and the use of these methods in the above-mentioned studies.

2. MATERIALS AND METHODS

In our experiments, Pannon White does and their kits were used. Parameters to characterise the processes of gastrointestinal tract (GI) and some production parameters were measured. Body weight and feed consumption was measured weekly and twice a week, respectively. Weight gain and feed conversion were calculated. Mortality was checked daily, while morbidity was assessed weekly.

All research protocols were approved by the Agricultural Administrative Authority (Protocol No. 00618/007/SOM/2003).

2.1. Effect of different weaning age

The aim of the study was to examine the effect of age and weaning on growth and certain parameters of the digestive tract in rabbits to assess the risk of early weaning for higher morbidity attributable to the presumably less developed digestive system.

The 1-day-old kits of average birth weight were distributed into litters of eight, and these litters were randomly divided into three groups according to weaning age – rabbits weaned at the age of 21 (G21), 28 (G28) or 35 (G35) days. Young rabbits were allowed to consume the diets, *ad libitum*. Milk intake of the litter was determined.

At 14, 21, 28, 35 and 42 days of age, six healthy animals from each group were randomly selected and narcotizes by CO_2 gas and sacrificed. The digestive system was removed and the caecum was separated. Samples were taken to laboratory analysis. The weight of the liver, heart, kidneys and lungs, as well as of the empty stomach, small intestine and caecum was measured. Relative weights of GI organs and emptied organs were calculated and expressed as a percentage of weight of GI organs.

2.2. Effect of *Bacillus cereus* var. *toyoi* (Toyocerin®)

The aim of the study was to examine the effect of a probiotic, *Bacillus cereus* var. *toyoi* on the composition and fermentation activity of the caecal microbiota around weaning. We examined also if there is any difference in the effect if kids consume the does' supplemented diet and so get the probiotic after the weaning.

One group of does (Group T) was fed a diet containing 0.05% Toyocerin (200 ppm, $2*10^5$ *Bacillus cereus* var. *toyoi* spores /g feed, Asahi Vet. S. A., Barcelona, Spain). The other group of rabbits (Group C) received an antibiotic-free diet with the same chemical composition.

Kits consumed the same diet as their mothers (Group C and T) till weaning. After weaning at age 28 days, all litters were divided into two groups, one feeding with the same diet as before, while the diet of the other two groups changed, so that half of Group C was fed with T supplemented feed, while half of Group T received control feed after weaning.

At the age of 21, 28 and 35 days, six healthy animals from each group were narcotized by CO_2 gas and sacrificed. The digestive tract was removed immediately and the caecum was separated. Samples were taken for laboratory analyses.

2.3. Effect of inulin supplementation

The aim of this experiment was to study the effect of age and dietary supplementation with inulin on growth performance and certain digestive physiological parameters, especially the caecal ecosystem and the fermentation in weaned rabbits.

According to the diet, the litters were randomly allocated into three groups at 21 day of age. A control diet (C) was formulated with no supplementation. A second diet (M) was obtained supplementing C diet with antibiotics. A third diet (I) was obtained 4% inluin (Frutafit, HD, Brenntag, Budapest) supplementation. At 28, 35 and 42 day of age 6 healthy animals from each group were randomly selected, narcotized by CO_2 gas and sacrificed. The digestive tract was removed immediately and the caecum was separated. Samples were taken for laboratory analyses.

2.4. *In vitro* metabolism of inulin by rabbit caecal microbiota

In our previous *in vivo* experiment, growing rabbits were fed non-medicated, medicated and inulin (4%) supplemented diet. Contrary to other results, there was no positive effect of inulin on production and caecal fermentation. Therefore, two *in vitro* experiments were carried out to analyse the effect of incubation the caecal content with inulin, on the composition of microbiota and volatile fatty acid (VFA) production.

In both experiments the rabbits (n=3) were fed with commercial fodder. Rabbits were 10 and 12 week old in experiment 1 and 2, respectively. The caecal content was homogenized, and divided into two portions. Sample 1 was control, while 4% inulin was added to Sample 2 directly. Samples were placed into culture dishes, in which the anaerobic conditions were ensured with the help of gasifying bag. Subsequently, the samples were incubated at 37^{o}C for 6 and 12 hours, respectively. Samples were taken after 0, 6 and 12 hours of incubation for laboratory analyses.

2.5. Laboratory analyses

The pH values of the fresh gastric, intestinal and caecal contents were measured by OP-110 pH meter (Radelkis, Hungary).

One gram of caecal digesta was used immediately after sampling for microbiological culturing technique. Number of total aerobic bacteria, *E. coli* and other coliforms and obligate anaerobic bacteria was determined. Incubation time had elapsed, and the colonies were counted according to standard with Acolyte colony counter. The colony counts were expressed in $\log_{10}$ colony forming unit (CFU) related to 1 g of sample.

The concentration of volatile fatty acids (VFA) was measured by gas chromatography (Shimadzu GC 2010, Japan).

The fibrolytic activity of the caecal bacteria was analysed by measuring the activity of cellulase, xylanase and pectinase.

In case of samples from inulin supplementation experiment, the total genomic DNA from caecal sample was extracted and purified with QIAamp® DNA Stool Mini kit. This extracts were utilized for Capillary Electrophorasis-Single Strand Conformation Polymorphism (CE-SSCP) and Real Time-Polymerase Chain Reaction (RT-PCR). Molecular genetics investigations were performed in INRA-TANDEM research institute, Toulouse, France.

2.6. Statistical analyses

Statistical analysis of the data obtained was carried out by Statistical Package for the Social Sciences (SPSS, 2002) version 10.0. One way Analyses of Variance (ANOVA) was used to analyse the data of weaning ages, probiotic experiments and RT-PCR. The statistical model of prebiotic experiments included diet, age and their interaction as main effects, which were studied by General Lineral Model (GLM). The significance of differences was tested by LSD and Tukey's post hoc test. Mortality and morbidity of the groups was compared by Chi-squared analysis.

3. RESULTS

3.1. Effect of different weaning age

By the age of 35 days, the body weight of animals weaned at 35 days of age (940 g) was higher value by 14 and 10% ($P<0.05$) as compared to those weaned at 21 and 28 days of age (826 and 850 g), respectively.

Early weaned animals had 75% higher feed intake compared to G28 and G35 rabbits ($P<0.05$) in the 4^{th} week.

Solid feed conversion increased from 1.4 g/g (between days 22 and 28) to 1.9-2.1 g/g and 2.6-3.0 g/g (between 29 to 35 and 36 to 42), respectively, without any significant difference being detectable between the groups.

The relative weight of GI tract according to weight of the rabbit was 5.5% at 14^{th} day of age, thereafter it was increased two times higher amount at the 42^{th} day of age in early weaned rabbit group. There were significant differences between groups on 28^{th} and 35^{th} day, but it was disappeared at 42^{nd} day of age.

The relative weight of empty stomach according to the weight of GI tract was decreased from 33% to 18% in case of early weaned rabbits. The ratio of small intestine weight was decreased from 44% to 33%, as well. The ratio of caecum was duplicated during the experimental period (from 10% to 20%). According to the calculation, the ratio of these organs was similar in case of the other groups.

Significant differences between groups were not found in the pH value of either the gastric or the caecal content. The pH value of the stomach content decreased to 1.6 between 21 and 42 days in all groups. The pH of the small intestinal content increased from 6.8 to 7.6 and 8.4 ($P<0.05$)

by day 21 and 28, respectively, in G21 rabbits. The caecal pH-value was decreased from 7.1 to 6.4 in the G21, between 14th to 21st day of age.

The strictly anaerobic bacteria were presented in high amounts (10^8) in the caecum, already at the age of 14 day. Their number increased ($\log_{10}$ 9.5), thereafter decreased to $\log_{10}$ 7.8 in G21 group ($P<0.05$). It this group, number of coliforms and total aerobic bacteria was decreased from $\log_{10}$ 2.8 to 1.9 and from 6.9 to 4.6, respectively between 14 and 42 day of age ($P<0.05$). The values of other groups were similar.

Throughout the experimental period, there was significantly higher tVFA production in G21, than in case of later weaned rabbits. The ratio of propionic: butyric acid (C3/C4) was decreased from 1.3 to 0.5 in G21 at 14th and 21st day of age, respectively. This value was significantly lower compared to the other groups (G28: 0.6-0.7 and G35: 0.5-0.6) between 28th and 42nd day of age.

3.2. Effect of *Bacillus cereus* var. *toyoi* (Toyocerin®)

Supplementation of the does' diet increased significantly the growth of the kits. On the 3rd week BW of T rabbits was significantly higher (462±12g), than BW of the C rabbits (389±8g). It was presumably due to the higher milk production of the T does and consequently, the better nutrient supply of the kids. The difference between BWs among groups was detectable still on the 4th week. Kids of T does (717g and 653g, respectively in group of TC, TT) had higher body weight than that of C does (610g and 543g, respectively in group of CC , CT).

Examining the whole experimental period, CT rabbits reached the highest BW (1301g) at 42 day of age and their feed conversion was the best as well (2.0 g/g).

Rabbits consuming supplemented diet after weaning had better health condition, than the other group rabbits (Table 1).

In the caecal content of dead rabbits, high number (10^6-10^7 CFU/g) of *E. coli* was detected. There was a significant difference in the number of coliforms, being higher in C rabbits ($\log_{10}$ 5.9), than in T group ($\log_{10}$ 4.3) on the 21st day of age. The difference disappeared to the 29th day of age, thereafter Toyocerin caused significantly less coliforms in groups CT and TT ($\log_{10}$ 2,0) at 35th day of age.

The coliform bacteria count was $\log_{10}$ 3-4 CFU/g chyme in group TC. It could be physiological value, but the count of $\log_{10}$ 5 in CC rabbits is considered to be of high risk from the animal health point of view.

Table 1: Health Risk Index after weaning (%)

Treatment	**Age (days)**	
	28-35	**36-42**
HRI* (%)		
CC	2.7	35.5
CT	0	6.20
TC	6.8	8.35
TT	0	2.85

*HRI=health risk index (the sum of morbidity and mortality)

3.3. Effect of inulin supplementation

The feed intake of rabbits fed inulin (I) was 11% lower value (76 g/day) compared to medicated (M) diet (85 g/day) ($P<0.05$). Growth rate from 28 to 35 days of age was not affected by type of diet, but rabbits fed I diet reduced it by 24% between 36 and 42 days of age.

Morbidity was higher in I group (3.3%, $P<0.05$), than in the other groups. No effect of age and treatment on mortality was observed. Diets had no effect on caecal pH, bacterial counts, and cellulase and pectinase activity.

Inulin diet decreased xylanase activity (by 18%, $P<0.05$) compared with C and M diets.

Total VFA concentration was not affected by diet, except in group C, where a temporary decrease (from 52 to 31 mmol/kg) by 40% was observed on 35 day of age ($P<0.05$).

Medicated diet caused lower proportions of acetic acid (75%) than C (80%) and I (82%) diets, and higher of propionic (9%) and butyric acids (12%) compared to I diet (7 and 9%, $P\leq0.05$).

A positive correlation between caecal counts of *E. coli* and caecal pH was observed throughout the experiment ($r = 0.32$, $P = 0.019$, $n = 54$), being highest value at 35 day ($r = 0.612$, $P<0.007$, $n = 18$).

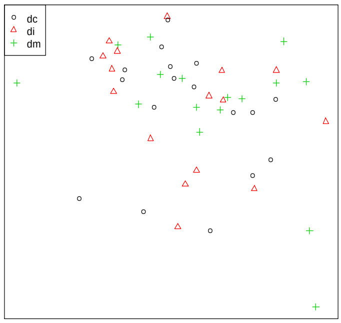
The number of the strictly anaerobic bacteria decreased by 10% and cellulase and xylanase activity increased by 25% and 29%, respectively ($P<0.005$) between 28 and 42 day of age.

With the help of the CE-SSCP technique some new qualitative information about rabbit caecal bacterial community has been determined. According to the principle of the fingerprint technique one bacterial species makes one peak in the profile. The tendency is that bacterial community more and more similar to each other in later ages. According to the nMDS plots, diet had no effect on bacterial community (Figure 1) as was shown by using the conventional culturing technique as well.

The concentration of propionic acid correlated significantly ($r = -0.407$, $p<0.01$) with the structure of community (Figure 2). The Simpson index showed also age ($p<0.001$), but no diet effect. The diversity index was increased from the beginning (4.6) to the end (5.2) of experimental period.

According to the results of RT-PCR measurements, there were significant differences between ages. Between days 28 and 42, the copy number of total bacteria and *Bacteroides-Prevotella* decreased by 2.5%

(from $\log_{10}$ 12.3 to 11.9) and 16% (from $\log_{10}$ 10.2 to 8.6), respectively. Even the ratio of *Bacteroides-Prevotella* was less by 0.1% within total bacteria represented in the caecum on day 42 compared to amount (1.3%) of day 28.



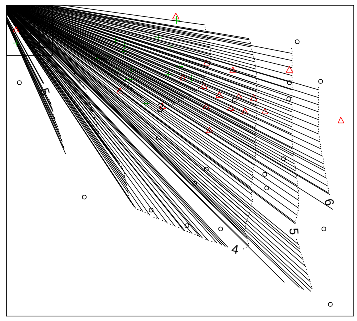


Figure 1: nMDS test - Effect of diet, 2D visualisation

dc: control -, di: inulin -, dm: medicated group

Figure 2: Relation between propionic acid and bacterial community

^{o} 28 days, ^ 35 days, + 42 days

Summarizing our data, 4% inulin supplementation of the feed between 21 and 42 days of age had no positive effect on caecal bacterial community, number of the measured bacteria, bacterial activity and the animal health status. The reason of invariable parameters could be the partially degradation of inulin in the upper GI tract. Inulin cause better growth of *Bifidobacteria*, which degradates carbohydrates intracellularly. In case of *Bacteroides* degradation is presumed to be periplasmic or extracellular, causing loss of digestion products. As an important part of the rabbit caecal microbiota consists of *Bacteroides* this result may also provide an explanation of the lack of effect of inulin supplementation.

To clarify the effect of inulin, we prepared two *in vitro* experiments without any side effect.

3.4. *In vitro* metabolism of inulin by rabbit microbiota

As the effect of incubation time, tVFA increased in experiments, by 75% (from 50.1 to 87.6 mmol/kg) and 126% (from 36.6 to 82.9 mmol/kg), respecively. The ratio of acetic acid slightly decreased (from 79% to 64%), while that of the propionic acid remained relatively constant (5-7%) in experiment 1 and increased in experiment 2. There was a significant increase in the butyric acid ratio (from 17 to 27%) in both experiments.

The total VFA production was lower in samples supplemented with inulin (60 mmol/kg) compared to control (86 mmol/kg), in experiment 1. The ratio of acetic acid and propionic acid was lower, while that of butyric acid was higher due to inulin supplementation.

In both experiments the number of the obligate anaerobe microbes slightly decreased by 7% (from $\log_{10}$ 7.5 to 7.0) and 5.5% (from $\log_{10}$ 7.6 to 7.2), respectively. There was an opposite change in the coliform count in the two experiments; it increased in experiment 1 by 4.6% (from $\log_{10}$ 4.4 to 4.6), while decreased by 9% (from $\log_{10}$ 4.4 to 3.9) in experiment 2. The number of the total aerobe bacteria slightly increased, but this change (from $\log_{10}$ 4.7 to 4.9) was significant only in experiment 1.

No consistent effect of inulin on caecal microbiota and fermentation could be detected in these two *in vitro* experiments. The differences between the two experiments can be probably attributable to the different age of the rabbits, taking into consideration that the animals consumed medicated diet till age of 9 weeks. Sampling happened one week after changing to non medicated diet in experiment 1, while the animals in experiment 2 had three weeks for adaptation.

4. CONCLUSIONS

In rabbit production, the time around weaning is the most crucial period, when rabbits are highly sensitive to multifactorial digestive disorders.

Weaning age significantly influenced the growth of rabbits. In traditionally weaned (35 day) rabbits, milk consumption and the additional solid feed intake resulted in better growth. The weaning age did not cause significant changes in the counts of some cultured bacteria, whereas it influenced VFA production. Early weaning (21 day) did not produce considerable changes in the digestive physiological parameters measured, but it resulted in reduced (by 10%) growth in rabbits. Among experimental conditions early weaning could be performed without the increase of morbidity or mortality, which suggests, that rabbits can be weaned on day 21 and reared without medication or feed additives under high hygienic and management conditions.

The supplementation of the diet with *Bacillus cereus* var. *toyoi* had a positive effect on production before weaning. This means that supplementation of the does’ diet was favourable regarding the growth of the kids, as they were allowed to consume the same diet between 21 and 28 days of age, i.e. till weaning. Although taking the whole experimental period into consideration body weight gain and feed conversion proved to be the best in group CT rabbits (consuming the supplemented diet only after weaning), which means, that they could compensate the slight lag in earlier weight gain after weaning.

Rabbits fed with the probiotic after weaning had significantly less coliform germ count in the caecum compared to the other animals. This could be the reason for the better health status (significantly lower morbidity and mortality) and growth.

According to the Commission Implementing Regulation (EU) No 288/2013 the products containing *Bacillus cereus* strains were withdraw from the food and feed production of European Union markets.

In our experiment 4% inulin supplementation decreased feed intake (by 11% compared to rabbits fed medicated diet). It decreased growth rate, while increased morbidity without influencing mortality. Consumption of inulin resulted in decreased caecal xylanase activity, reduced propionic and butyric acid while increased acetic acid production. No effect on the composition of the microbiota could be detected. The classical culturing microbiological results were confirmed by RT-PCR technique.

No consistent effect of inulin on caecal microbiota and fermentation could be detected in the *in vitro* experiments as well. Inulin supplementation resulted in lower tVFA production but higher butyric acid ratio. The number of the aerobe and obligate anaerobe bacteria decreased, while the change in coliforms was not consistent in the two experiments.

Bifidobacteria are not dominant in rabbit ceacum, but an important part of the rabbit caecal microbiota consists of *Bacteroides,* which seemed to be neither stimulated nor depressed through administration of inulin. So this kind of prebiotic can not fully propose in rabbit meat production, more investigations into its mode of action and interaction with rabbit microbiota is needed.

Our results may facilitate the reduction of antibiotic use during rabbit growing and, eventually, contribute to the full replacement of antibiotics by alternative solutions in rabbit production.

5. NEW SCIENTIFIC RESULTS

1. Early weaning (at 21 days of age) resulted in lower (10%) growth, earlier growth of the gastrointestinal tract, more short chain fatty acid (SCFA) production, acetic acid and butyric acid proportion, while less propionic acid ratio between 35-42 day of age in caecum of Pannon White rabbits.

2. Age related changes influenced the relative weight of the liver, the gastrointestinal tract, the relative weight of the stomach, small intestine and caecum within the GI tract, as well as the relative weight of the GI content, regardless of the weaning age.

3. Age related changes affected the number of total bacteria and *Bacteroides-Prevotella* imagined by CE-SSCP and determined by qPCR, i.e. decreased (by 60.2 and 97.5%, respectively), in caecal content samples, between days 28 and 42. The ratio of *Bacteroides-Prevotella* group within total bacteria represented in the caecum was less by 1% on day 42 compared to data of day 28. The amount of bacteria not belonging to *Bacteroides-Prevotella* group was increased by 2% (from 97% to 99%) between days 28 and 42.

4. Supplementation of the does' diet with *Bacillus cereus* var. *toyoi* significantly increased (by 19%) the growth of the kits. Rabbits fed a diet supplemented with this strain of bacterium after weaning had significantly better health condition and less coliform bacterial count in the caecum (by 65 and 250%, respectively), compared to rabbits fed a diet not supplemented by this probiotics after after weaning.

5. Supplementation of the diet with 4% inulin had no positive effect on growth, decreased feed intake (by 11%), and resulted in a higher morbidity and mortality. Inulin supplementation of the diet decreased xylanase activity (by 18%) in the caecal content, and as a result lowered the molar proportion of butyric acid, while did not influence caecal microbiota determined by culturing, CE-SSCP and RT-PCR.

6. SCIENTIFIC PAPERS AND LECTURES ON THE SUBJECT OF THE DISSERTATION

6.1. Peer-reviewed papers published in foreign scientific journals

M. Kovács, Zs. Szendrő, G. Milisits, B. Bóta, E. Bíró Németh, I. Radnai, R. Pósa, A. Bónai, F. Kovács, P. Horn.: Effect of nursing methods and faeces consumption on the development of the bacteroides, lactobacillus and coliform flora in the caecum of the newborn rabbits. Reproduction, Nutrition, Development, 2006. 46:(2) 205-210. (ISSN: 09265287) (IF=1.817)

A. Bónai, Zs Szendrő, Zs. Matics, H. Fébel, L. Kametler, G. Tornyos, P. Horn, F. Kovács, M. Kovács: Effect of inulin supplementation and age on growth performance and digestive physiological paremeters in weaned rabbits. World Rabbit Science, 2010. 18:(3) 121-129. (ISSN: 12575011) (IF= 0.660)

M. Kovács, A. Bónai, Zs. Szendrő, G. Milisits, H. Lukács, J. Szabó-Fodor, G. Tornyos, Zs. Matics, F. Kovács, P. Horn: Effect of different weaning age (21, 28 or 35 days) on production, growth and certain parameters of the digestive tract in rabbits. Animal, 2012. 6:(6) 894-901. (ISSN: 17517311) (IF=1.744)

6.2. Peer-reviewed paper published in Hungarian scientific journal

Bónai A., Szendrő Zs., Matics Zs., Fébel H., Pósa R., Tornyos G., Horn P., Kovács F., Kovács M.: Effect of Bacillus cereus var. toyoi (Toyocerin®) on caecal microflora and fermentation in rabbits (in Hungarian). Magyar Állatorvosok Lapja, 2008. 130:(2) 87-95. (ISSN: 0025-004X) (IF=0.088)

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M. Kovács, G. Milisits, Zs. Szendrő, H. Lukács, A. Bónai, R. Pósa, G. Tornyos, F. Kovács: Effect of different weaning age (days 21, 28 and 35) on caecal microflora and fermentation in rabbits. In: Xiccato G, Trocino A, Lukefahr SD (szerk.) Proceedings of the 9th World Rabbit Congress. Verona, Olaszország, 2008.06.10-2008.06.13.
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A. Bónai, Zs. Szendrő, L. Maertens, Zs. Matics, H. Fébel, L. Kametler, G. Tornyos, P. Horn, F. Kovács, M. Kovács: Effect of inulin supplementation on caecal microflora and fermentation in rabbits. In: Xiccato G, Trocino A, Lukefahr SD (szerk.) Proceedings of the 9th World Rabbit Congress. Verona, Olaszország, 2008.06.10-2008.06.13.
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Brescia: 561-566. (ISBN: 9788890281464)

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International Symposium on Housing and Diseases of Rabbits, Furbearing Animals and Pet Animals, Celle, Németország, 2009. 05. 13-14. 80-87. (ISBN 978-3-941703-04-9)

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