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EXAMINATION OF THE EFFECTS OF CERTAIN FACTORS INFLUENCING CAECAL FERMENTATION IN RABBITS

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LIST OF ABBREVIATIONS

ANOVA	**Analyses of Variance**
ADF	**Acid Detergent Fiber**
ADL	**Acid Detergent Lignin**
BW	**Body Weight**
CE	**Capillary Electrophoresis**
CP	**Crude Protein**
CFU	**Colony Forming Unit**
d	**Day**
DE	**Digestible Energy**
DM	**Dry Matter**
DNA	**Deoxyribonucleic Acid**
FAO	**Food and Agriculture Organization**
GI	**Gastrointestinal**
GLM	**General Lineral Model**
h	**Hour**
HRI	**Health Risk Index**
ISO	**International Organization for Standardization**
LSD	**Least Significant Difference**
NDF	**Neutral Detergent Fiber**
nMDS	**non-metric Multi Dimensional Scaling**
PCA	**Principal Component Analysis**
RDA	**Redundancy Analysis**
RT - PCR	**Real Time - Polymerase Chain Reaction**
SSCP	**Single - Strand Conformation Polymorphism**
SCFA	**Short Chain Fatty Acid**

1. INTRODUCTION

1.1. Antecedents

During the past 50 years, rabbit meat production of the world has quadrupled. In 2010, global rabbit meat production was approximately 1.7 million tons, and this production volume showed the following distribution by continent: Asia 48%, Europe 30%, America 17%, and Africa 5%. According to data from the FAO (2012), the biggest rabbit meat producing countries of the world were China (668,980 tons), Italy (255,420 tons) and Venezuela (254,305 tons).

Based on data of the Hungarian Rabbit Product Council, in 1990 the quantity of rabbit meat production in Hungary was 33,468 tons (liveweight), of which 16,763 tons were exported. Over the past 20 years, both values have decreased to about one-third. Thus, in 2011 the quantity of rabbit meat production in Hungary was 10,300 tons (liveweight), of which 5090 tons were exported. Hungary has about 60–65 large rabbit farms with approximately 85000 does. At the two rabbit slaughterhouses (Baja and Lajosmizse) about 70–75 thousand rabbits are slaughtered weekly, in approximately equal proportions (Jurasko, 2013).

The rabbit sector of Hungary is unique in the world in that almost the entire quantity of meat from rabbit production for slaughter (the price of rabbit meat was 420–450 HUF/kg in 2012) is exported. The most important export markets of Hungary for rabbit meat are Italy, Switzerland and Germany.

The advantages to grow rabbits are as follow: rabbits have a high fertility rate with a rapid rate of growth, high feed conversion efficiency and early marketing age and high muscle-bone ratios; in addition, they require a small land area (Fernandez-Espla and O'Neil, 1993). The significance of

broiler rabbit production is increasing for both healthy nutrition of humans and economic reasons.

Rabbit meat is considered to be one of the healthiest meats because of its easy digestibility and excellent dietetic properties, e. g. high protein (20–21%) and unsaturated fatty acids (oleic and linoleic acid; 60% of all fatty acids), potassium, phosphorus and magnesium concentrations and also low fat, cholesterol and sodium contents (Hermida et al., 2006). The rabbit meat is useful in human dietetics and recommended for consumption, e.g. to persons suffering of cardiovascular diseases (Hu and Willett, 2002). According to Dalle Zotte (2002), the energy value of rabbit meat (427–849 kJ/100g of fresh meat) is similar to the values of commonly consumed meats, such as pig (418-1121 kJ/100g), beef (473-854 kJ/100g) and chicken (406-808 kJ/100g) (Dalle Zotte, 2002).

Diseases affecting the digestive tract cause major problems during the growing period of broiler rabbits. These diseases are often severe and may be fatal. About one-fourth of all mortality takes place in the period around weaning (at 2–6 weeks of age). This gives cause for concern from the animal welfare point of view and causes a substantial loss of profits to the rabbit growers. In order to minimise the losses, antibiotics are used on a wide scale, which results in a major food safety and human health risk.

From the middle of the past century, the development of intensive animal breeding made it necessary to use antibiotics as growth promoters. Their use resulted in the improvement of many production parameters; therefore, from the 1970s the use of antibiotic growth promoters became widespread all over the world. Besides its benefits, antibiotic use also has detrimental consequences. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a significant infection-causing bacterium, since it gives rise to potentially life-threatening infections and also shows resistance to treatment

with the usual antibiotics. Hospital-acquired MRSA infections are associated with increased morbidity and mortality (Engemann et al., 2003). The incidence rate of MRSA is less than 1% in the Northern European countries while it exceeds 40% in the Southern and Western European countries. The proportion of human MRSA infections is decreasing in some countries but increasing in others (Anonymous, 2008).

Due to the risk that potentially pathogenic microbes develop resistance to antibiotics, the European Union has restricted the possibilities of using antibiotics. Antibiotics authorised for use in human medicine have been separated from those permitted for use in veterinary medicine. Finally, with effect from 1 January 2006, the growth-promoting use of all antibiotics applied in animal production, with the exception of anticoccidials, was banned on the territory of the European Union. This has resulted in an increased demand for alternative growth promoters (Ancsin et al., 2008).

This regulation applies to the animal production sector of Hungary as well, as Hungary has been a member of the European Union since 2004. 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 higher occurrence of deseases, and researchers have to find new solutions suitable for replacing the use of antibiotics. One way to this latter objective, it would be important to get to know the bacteria living in the digestive tract of rabbits and their metabolic processes as thoroughly as possible.

In association with a series of experiments underway at the Department of Physiology and Animal Hygiene, I studied the problems related to the development of diseases in the period around weaning in domestic rabbits, and looked for solutions suitable for replacing the use of antibiotics during

the growing period in rabbits. I determined the effect of weaning age on the implatation of digestive microbiota, and conducted experiments to determine the effects of probiotics and prebiotics added to the diet.

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.

1.2. Objectives

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. REVIEW OF THE LITERATURE

2.1. Characteristics of the digestive tract and digestion of rabbits

During evolution, the digestive tract of the domestic rabbit has adapted to the herbivorous lifestyle. As a consequence, the volume of the stomach and caecum has increased considerably compare to the upper digestive tract.

The functioning of the rabbit's upper digestive tract globally is the same as that of other monogastric domestic mammals. The speciality of the rabbit (and of *Lagomorpha* in general) lies in the dual function of the proximal colon (Gidenne and Fortun-Lamothe, 2002). Bacteria stimulate the immune system of rabbit, and bacteria enzymes ensure the degradation of forage fibers.

The rabbit stomach has a weak muscular layer and is always filled partially. Soft faeces is stored in the fundic region of the stomach after caecotrophy. The pH value depends on the region of stomach, in the fundus and in the cardiac-pyloric region it is about 1 and 5, respectively (Chamorro et al., 2007; Gomez-Conde et al., 2009).

In an adult rabbit the small intestine is approximately 3 m long, where the secretion of bile, digestive enzymes and buffers occurs. The pH value of the small intestine is close to 7 (Nicodemus et al., 2002). Digestibility at the end of the ileum accounts for 80-100% of the total dietary amino acid and starch digestibility (Carabano et al., 2009).

The caecum is the largest digestive compartment of the rabbit (40% of the whole digestive tract). It plays a key role in the digestion as a major site of fermentation (e.g. fibre degradation). For instance, in adult rabbits, SCFA absorption could represent 30% of the basal metabolism (Parker, 1976; Marty and Vernay, 1984).

The caecum is characterised by a weak muscular layer and content with a dry matter of 200 g/kg. The caecal contents are slightly acidic (pH 5.4–6.8) (Garcia et al, 2002). The capacity of the caecum is approximately 49 % of the total capacity of the digestive tract (Portsmouth, 1977). There is evidence that the caecal microbial activity plays a key role in the digestion and health of the rabbit (Gidenne, 1997).

The colon can be divided into the proximal and distal colon (approx. 35 and 80 cm long, respectively). The rear section of the distal colon acts as a pacemaker for the colon during the phase of hard faeces formation (Snipes et al., 1982).

2.1.1. Microbial balance in the intestine

The normal intestinal microbiota has characteristic composition which, however, differs by animal species, age and intestinal segment. It is constituted approximately by 400–500 species, in a total microbial count ($\log_{10}$ 14 CFU/g chyme) in the intestinal tract of animals (Gedek, 1991). The digestive tract of healthy animals is colonised only by microbes typical of the given animal species and age. Between the host and the microflora a regulated state of equilibrium, called eubiosis, is developed (Szigeti, 1991).

Gouet and Fonty (1979) showed that 75% of the young rabbits do not harbour any flora in the stomach during the first 15 days of life, in spite of the existence of a slightly acidic pH (4.5 to 5.0). Barrier function of stomach is realized by fermentation end-product of *Lactobacillus* species. Number of pathogenic bacteria originated from feed is reduced by antiseptical lactic acid.

The number of bacteria is further reduced due to octanoic and decanoic acids of the rabbit doe milk in the gastrointestinal tract during the

suckling period (Canas-Rodrigues and Smith, 1966; Coloe et al., 1984; Marounek et al., 1999, 2003).

Around the 3rd week of age, young rabbits start to consume solid feed, and caecotrophy begins as well. After weaning, the level of the gastric flora is $\log_{10}$ 4 - 6 CFU/g of digesta (Raibaud et al., 1966).

Colonisation of the small intestine takes place faster than that of the stomach, and already in the first week of life the bacterial count in the small intestine is approx. 100 times higher than that in the stomach. Facultative anaerobic bacteria are still detectable in the small intestine before weaning but usually disappear from it thereafter (Gout and Fonty, 1979).

In the first week of life, the quantity of the caecal microbiota is $\log_{10}$ 7 - 9 CFU/g of caecal content. After weaning, the count of facultative anaerobic bacteria decreases to $\log_{10}$ 2 - 4 CFU/g of content (Ducluzeau, 1983). The number of obligate anaerobic bacteria becomes stabilised on the level of $\log_{10}$ 9 - 10 CFU/g of digesta (Zomborszky-Kovács et al., 2000).

Those bacteria can participate in the constitution of the intestinal microflora which can attach to the surface of epithelial cells, and which are multiplicated at a faster rate than are removed by intestinal peristalsis (Ewing and Cole, 1994). One of the most important beneficial effects of the indigenous gastrointestinal microflora is to make the exogenous pathogenic bacteria more difficult to colonise the digestive tract (Berg, 1996). Competitive exclusion is a complex inhibition process, which means that a non-pathogenic species predominates over a pathogenic one. Competition can be realised by differences in growth on a specific substrate, efficiency of mucosa colonisation, and production of substances inhibiting pathogen development (Hampson et al., 2001). The surface binding sites of intestinal epithelial cells can also be occupied by certain natural receptor analogues (e.g. oligosaccharides). In this way, enteropathogenic bacteria will not be

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able to attach to the surface of intestinal epithelial cells and thus become members of the transient microbiota (Kelly and Tucker, 2005).

Bacteria can inhibit the growth of their competitors also by producing antimicrobial substances (Guarner and Malagelada, 2003).

Acting as antigen, the live and the already perished microbes stimulate the mucosal immunity of the digestive tract (GALT) and elicit enhanced antibody (sIgA) synthesis as well as T and B cell activity (Ewing and Cole, 1994).

2.1.2. Characteristics of the caecal microbiota

Bacteroides bacteria can be found in the caecal microbiota of calves, lambs and piglets. Their number decreases with age, and in adult animals they can no longer be detected (Smith and Crabb, 1961).

In caecum of newborn colt, there are detectable species of the *Clostridium*-, *Enterococcus*-, *Enterobacter*-, *Lactobacillus*-, *Streptococcus*-, *Staphylococcus* genus (Jullian et al., 1996). Initially, members of *Enterococcus*- and *Enterobacteriaceae* genus are dominant (Yuyama et al., 2004). Members of *Bacteroidaceae*, *Clostridium*, *Enterobacteriaceae* ($\log_{10}$ 8.3 CFU/g) and *Enterococcus* ($\log_{10}$ 7.7 CFU/g) were founded in faeces of three day old colt (Sakaitani et al., 1999).

Genus of *Bacteroidaceae*- and *Lactobacillus* is dominated in colt faeces, numbered $\log_{10}$ 8.0 CFU/g digesta, from the 6th week of life. Number of *Enterobacter*-, *Enterococcus*-, and *Staphylococcus* bacteria is around $\log_{10}$ 5.0 CFU/g (Jullian et al., 1996; Sakaitani et al., 1999).

In rabbits the bacteria number of *Bacteroides* is stabilised on a high level and they become constituents of the main microflora (Smith and Crabb, 1961). This has been confirmed by the results of Gouet and Fonty (1979), Boulahrouf et al. (1991) and Carabano et al. (2006) as well. However these

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results have been queried with the introduction of molecular approach methodologies (Abecia et al., 2005; Combes et al., 2011).

According to Fuller and Lev (1964), caecal microbiota constituent characteristic of other domestic animal species, among others *Bifidobacteria*, can not be found in the rabbit caecum. Bornside and Cohn (1965), as well as Smith (1965) described a steady composition of the microbiota present in the digestive tract of adult domestic rabbits. This is characterised by the almost complete absence of lactobacilli, streptococci and *Escherichia coli* strains abundantly present in the gastrointestinal microflora of other species (Gouet and Fonty, 1973).

Although more than 74 strains of anaerobic bacteria have been isolated from the caecal mucosa of domestic rabbits, *Bacteroides* bacteria were regarded as the main constituents of the microbiota for a long time (Cheeke, 1987; Straw, 1988).

The cellulolytic bacterial community appears around 2 weeks after birth. This community was found to be dominated by *Eubacterium cellulosolvens* and *Bacteroides* spp. (Boulahrouf et al., 1991).

According to Gidenne (1997), the two main characteristics of the caecal microflora in domestic rabbits are its slow development and stabilization, simple composition. The latter means the dominance of Gram-negative, non-spore-forming, strictly anaerobic, rod-shaped bacteria.

According to Harcourt-Brown (2002), a typical feature of the rabbit intestinal microflora is the very simple composition of the facultative anaerobic flora. Up to the 14th day of life streptococci are dominant, but their number rapidly decreases after weaning. Enterobacteria can hardly be detected at all, and lactobacilli are almost entirely absent as well.

Microbiological culture methods can be used successfully for only a few groups of bacteria (Rastall and Maitin, 2002). According to Suau et al.

(1999), only 20–40% of bacteria living in the caecum can be cultured. Thanks to the methodological developments involving the widely spreading genetic analyses and the experiments using such methods, the scope of information available on the caecal microbiota of rabbits is expanding fast. By using dot-blot hybridisation with 16S rRNA targeted oligonucleotides probes, Bennegadi et al. (2003) stated that bacteria and archaea respectively represent 73% and 22% of the total microbial communities in the caecum at weaning, however this result was not confirmed nor by counting nor by methanogenic activity. Bacteria of the Flexibacter – Cytophaga – Bacteroidetes group and four cellulolytic bacterial strains (*R. flavefaciens, R. albus, F. succinogenes, F. intestinalis*) were found.

Sequence and cluster analysis showed that more than 80% of the bacteria inhabiting the rabbit caecum are unknown, and bacteria belonging to the Prevotella/Bacteroidetes groups were not found either (Abecia et al., 2005). Subsequent, more accurate microbial genomic studies demonstrated that bacteria belonging to the Firmicutes (94%) and Bacteroidetes (4%) groups could be found in the bacterial community (Monteils et al., 2008). The maximal density of the Bacteroides-Prevotella group was reached 3 weeks after birth (log_{10} 10–11), but it decreased significantly thereafter (log_{10} 9–10) (Combes et al., 2011).

Michelland et al. (2010) studied the bacterial ecosystem of the rabbit caecum by Capillary Electrophoresis Single-Strand Conformation Polymorphism (CE-SSCP) technique, and found that there were no marked individual differences among rabbits in terms of the bacterial community present in the caecal content, and that the bacterial community of the caecal content was more similar to that of the soft than that of the hard faeces. At the same time, the composition of the bacterial community and the amount

of bacteria were markedly influenced by changes in dietary fibre content (Michelland et al., 2011).

2.1.3. Metabolic products of the caecal microbiota

Although rabbits do not possess enzymes necessary for degrading fibre, their caecum contains large numbers of fibrolytic bacteria that adhere to the surface of dietary fibre substances and start to brake down them with the help of their enzymes. Cellulose, pectin, xylan and amylose molecules are broken down into their components. Released monosaccharide molecules are taken up by the microbes and used during fermentation. The end-products of this process are short-chain fatty acids (SCFA), which cannot be degraded further under anaerobic conditions.

Ammonia is used by bacteria, in combination with carbon chain produced from carbohydrate fermentation, to synthesise new amino acids for bacterial growth (Van Soest, 1994).

The caecal metabolism of nutrients is similar in rabbits to that can be shown in other herbivores, but the SCFA pattern exhibits some differences in rabbits, namely a predominance of acetate, followed by butyrate and then by propionate (Gidenne et al., 2008). In an average, the relative proportions of individual volatile fatty acids within the total volatile fatty acids are as follow: 75–85% acetic acid, 6–10% propionic acid and 8–17% butyric acid (Fortun-Lamothe and Gidenne, 2006; Combes et al., 2011). The propionic acid to butyric acid ratio decreases below 1 by 25–30 days of age (Zomborszky-Kovács et al., 2000). The ammonia concentration of the caecal content slightly decreases with age (Gidenne and Fortun-Lamothe, 2002).

Feed composition, such as dietary fibre, interacts with the digestive health of the young animal (Montagne et al., 2003) and particularly of the

growing rabbit after weaning (Gidenne, 2003). The ratio of butyric acid usually increases substantially with the decreasing ratio of fibre and starch (Gidenne, 1997). Higher ratios of digestible fibre will lead to a larger volume of caecal content. Microbial activity results in the production of more volatile fatty acids and less ammonia, which lower the pH of the caecal digesta between 15 and 42 days of age (Gidenne and Fortun-Lamothe, 2002).

From 15 to 50 days of age, the pH of the chyme decreases from 6.8 to 5.6 (Padilha et al., 1995). The pH of the caecal digesta decreases from 6.2 at 28 days of age to 5.8 at 70 days of age (Kimse et al., 2009; Combes et al., 2011), though to evaluate these results, it is necessary to consider the effect of diet composition, as well.

2.1.4. Caecotrophy

Young rabbits start to eat a notable amount of dry feed at 3 weeks of age (Lebas, 1997), in connection with which caecotrophy commences between 22 and 28 days of age (Orengo and Gidenne, 2007).

The caecum is starting to be filled by digesta and microbiota from 3 to 7 weeks of age, and its contents reach a peak of about 0.06 part to total body weight at 7–9 weeks of age (Padilha et al., 1995).

The co-ordinated operation of caecum and colon results in fractionation of the intestinal content. During this process, fermentable particles less than 0.1 mm in size with higher nutrient content become concentrated in the caecum while the less valuable feed particles exceeding 0.3 mm in size accumulate in the colon. Rabbits excrete the larger particles definitively in the form of hard faecal pellets (Lebas et al., 1997).

The motility of the basis of caecum and the proximal colon decreases during the formation of soft faeces (Ruckebush and Hornicke, 1977), because of the effect of prostaglandin $F_{2\alpha}$ (Pairet et al., 1986).

Soft faecal pellets (small pellets of 5 mm size) covered by a mucous envelope from the proximal colon are taken directly from the anus and swallowed by the rabbit. They are stored (for 3–6 hours) in the fundic region of the stomach till digestion and absorption (Gidenne and Poncet, 1985).

The caecotroph is excreted according to a circadian rhythm. Monophasic pattern of soft faeces excretion was showed by rabbits between 8:00 and 17:00. During the caecotrophy period, lasting from 7:00 to 9:00, there is an absence of hard faeces excretion and the feed intake is low (Carabano and Merino, 1996).

After weaning, soft faeces production linearly increases with age, reaching a maximum (25 g DM/day) at 63–77 days of age. Thereafter, this value is stabilised around 20 g DM/day (Gidenne and Lebas, 1987).

Some data on the chemical composition of soft faeces, expressed as average values, are as follow: dry matter (340 g/kg), crude protein (300 g/kg DM), crude fibre (180 g/kg DM). Compared to soft faeces, the hard faeces has higher values of dry matter (470 g/kg) and crude fibre (300 g/kg), however, its crude protein content is lower (470 g/kg) (Carabano et al., 1997). The soft faeces contains essential amino acids (e.g. lysine and threonine) (Garcia et al., 2004). The main benefit of caecotrophy is the utilisation of proteins, fatty acids and vitamins of bacterial origin, and the decrease of metabolic losses (Meyer et al., 2010).

2.2. Anatomical and digestive changes associated with growth in rabbits

2.2.1. Growth and enzyme activity

The anatomy of the digestive tract is stabilised by 9 weeks of age. Between 3 and 11 weeks of age, the weight of organs is multiplied by 4 and 8 times for stomach and small intestine and for caecum and large intestine, respectively. Length is multiplied by 2 to 3 times for all digestive tract segments between 3 and 11 weeks of age (Lebas and Laplace, 1972; Alus and Edward, 1977; Xiccato et al., 2001).

In the first two weeks of life, newborn rabbits consume almost exclusively milk. The stomach can take up and store a large volume of milk. Between 1 and 3 weeks of age, the daily milk intake of rabbit kits increases from 10 g to 30 g per capita (Gidenne and Lebas, 2006). Stomach weight increases from 17 days (3.6 g) to 35 days of age (10.4 g) (Orengo and Gidenne, 2007).

In that period, the pH value of the gastric digesta ranges around 4.5–5.0. After the second week of life, when the milk production of the pregnant doe decreases, the young rabbits eat an increasing amount of solid feed. Hydrochloric acid secretion of gastric glands is stimulated by solid feeding components, therefore the pH value of the stomach content decreases to values around 1.5–2.0 (Fortun-Lamoth and Gidenne, 2006).

In the stomach of young rabbits, the proteolytic activity is provided by rennin at birth (Henschel, 1972) and by pepsin from 2 weeks after birth (Dojana et al., 1998). The proteolytic activity of the pancreas increases with age (Marounek et al., 1995).

During the suckling period, gastric lipase represents most of the lipolytic activity of the whole digestive tract, whereas this activity is not detectable in the 3-month-old rabbit (Marounek et al., 1995). Intestinal

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lactase activity is high until 25 days of age. The activity of sucrase and maltase rises until reaching the adult level (Gallois et al., 2008).

The weight of the pancreas increases greatly when the rabbit begins to eat solid feed (Lebas et al., 1971). Enzymatic activity of the rabbit pancreas increases progressively around days 21–24 of age, independently of the nature of diet (Corring et al., 1972). From 25 to 42 days of age, total enzymatic activity in the intestinal content strongly increases for chymotrypsin (5-fold), lipase (10-fold), amylase (17-fold) and maltase (11-fold), while trypsin activity is augmented only 2-fold between day 32 and day 42 (Gidenne et al., 2007).

Caecal fibrolytic activity is not detectable in young rabbits of 2 weeks of age. Cellulolytic activity improves progressively, reaches its maximum level around 35 days of age and stabilises thereafter. Xylanase and pectinolytic activity seems to increase between 10 and 24 weeks of age (Pinheiro et al, 2001). At 2 weeks of age, the amylolytic flora is active and stabilises between 15 and 49 days of age (Padilha et al, 1995).

The development of bacterial activity depends mainly on nutrients entering the caecum and consequently on dietary composition and digestibility (Gidenne and Fortun-Lamothe, 2002). The fibrolytic potential of the caecal flora is already high before weaning, even if fermentative activity remains weak (Gidenne et al., 2007).

A possible solution to reduce periweaning mortality may be the method of early weaning. As a result of early weaning, the weight of organs and organ contents increases, microbial colonisation is accelerated, fermentation activity is augmented and the maturation of the intestinal immune system becomes faster (Piattoni and Maertens, 1999; Gutierrez et al., 2002; Xiccato et al., 2003; Gallois et al., 2005; Carabano et al., 2008).

2.2.2. Development of the caecal microbiota

The stable microbial community of a given intestinal segment is established by bacteria having the ability to adapt to the conditions prevailing there and adhere to, and grow on, the surface cells of the intestinal mucosa. Numerous complex phenomena (bacterial motility, chemotactic as well as specific and nonspecific attraction) play a role in the mechanism of bacterial adhesion, during which the fimbria-type projections of the bacterium bind to the receptors of the intestinal epithelial cells. Colonisation requires the availability of properly defined receptor structures in a given animal species or even in individuals of a specific age or developmental status (Kelly and Tucker, 2005).

Colonisation means the constant presence and growth of a certain bacterial strain in a given segment of the digestive tract. In order to be regarded as a bacterium having colonised the intestine, a bacterial strain must be present in the intestinal microflora for a period of at least three weeks (Collignon and Adlerbecht, 2000).

Soon after birth, the microbiota must ultimately develop from a simple and unstable community into a complex and stable climax community in adulthood (Mackie et al., 1999).

According to the observations of Canas-Rodriguez and Smith (1966), the gastric microbiota is scanty in the first three weeks of life, which is probably attributable to the fatty acids of antibacterial effect present in the milk of the rabbit doe. This observation was supported by the findings of Marounek et al. (1999) as well.

The observations of Hudson et al. (1996) suggest that ingestion of the maternal faecal pellets may have an important role in the development of the intestinal microflora in newborn rabbits. According to these authors, in the second week of life rabbit pups start to intensively ingest the faecal pellets

left behind by the doe in the nest after nursing, and they eat some of the nest materials as well. The purpose of this phenomenon may be the quickest possible development of the intestinal microbiota. In addition, owing to their high fibre content the maternal faecal pellets provide an excellent growth medium for beneficial microbes capable of utilising dietary fibre.

Our previous studies (Kovács et al., 2002, 2004) have shown that Bacteroidetes bacteria are present in large numbers already when rabbit pups feed exclusively on milk and there is yet no fermentation in their caecum. We also studied the effect of maternal faeces consumption and the nursing method on colonisation of the caecum with *Bacteroidetes* (Kovács et al., 2006). Colonisation by bacteria begins already on day 3, independently of the nursing method and access to maternal faeces. On the 2nd day of life, total germ count was still below $\log_{10} 2$ CFU/g of digesta, while on the 4th day of life it was already between $\log_{10} 2 - 4$ CFU/g of caecal content. In freely nursed rabbit pups and in those having access to maternal faeces, colonisation by *Bacteroides* took place more rapidly. Prevention of the ingestion of maternal faeces only delayed the development of normal intestinal microflora. The fact that *Bacteroides* bacteria could be cultured from the surface of the doe's genital organs indicates that young rabbits could be infected by these bacteria already in the birth canal of does. Thus, colonisation of the intestine by the studied bacteria is not exclusively determined by the faeces in the nest environment. Therefore, it can be concluded that the characteristic components of the caecal bacterial ecosystem colonise the caecum already very early. During the period of exclusive milk feeding, their probable role is to develop the earliest possible protection against pathogens and to support intestinal development.

2.3. Eubiosis and dysbiosis in the intestine

The co-existence of the host animal and the intestinal microbiota in symbiosis, with the least possible burden posed to the intestinal immune system, can be defined as eubiosis. The intestinal microflora can be divided into the main, the satellite and the residual flora. In such a state of dynamic equilibrium, at least 90% of the total intestinal microflora is comprised by the main flora (including *Bacteroides* genus), 1−10% by the satellite flora (e.g. *Enterococcus* and *E. coli*), and less than 0.01% by the residual ones (e.g. members of the genera *Salmonella, Campylobacter, Staphylococcus* and *Clostridium*). Achieving and maintaining a state of eubiosis are of fundamental importance for keeping feed utilisation, production and health status on a desirable level. When the ratio of the satellite and residual flora to the main flora increases, eubiosis ceases and dysbiosis (dysbacteriosis) develops, which provides an increased burden on the intestinal immune system (Szigeti, 2003).

Dysbiosis can be induced by inadequate feed manufacturing technologies, poor hygienic condition of the feed, abruptly implemented feed changes, reduced intestinal motility, relative deficiency in gastric acid secretion, certain environmental stressors (e.g. weaning, temperature, transfer or regrouping), and pathogens taken up from the environment.

Young animals are especially susceptible to diseases triggered by disrupted balance of the microbiota, as initially the composition of the intestinal microflora is not stable yet. In the life of an animal, the days immediately after birth (the time of microflora development) and the time around weaning are the most critical periods with regard to the development of dysbiosis.

Weaning from the mother and the associated feed change represent a major stress for young animals. At that time, the composition of the

intestinal microflora detectably changes: the number of lactobacilli decreases while the counts of coliforms and *Escherichia coli* increase due to the lack of medium-chain fatty acids originated from the milk (Skrivanova and Marounek, 2006).

The pathogenic microbes (e.g. *Clostridium-*, *Salmonella-*, *Campylobacter* sp., *E. coli*) growing abundantly under such conditions can cause a wide variety of diseases resulting in an increased mortality rate. Of the different age groups, 4 to 6 weeks old rabbits are at the highest risk of developing digestive diseases.

After weaning, rabbit kits have to switch over to the exclusive consumption of solid feed. Although from about 3 weeks of age rabbit kits tend to eat also from the diet of their dam, and the adaptation of the intestinal microbiota takes time. The change of feed disrupts the balance of the microbial population that had until then been adapted to milk feeding. If the pathogenic bacteria find favourable conditions in the digestive tract and can adhere to the surface of the intestinal epithelial cells, they may propagate rapidly and cause disease in the host animal.

Depending on the original composition of the intestinal microflora, sometimes even the therapeutic doses of some broad-spectrum antibiotics (e.g. ampicillin and lincomycin) may disrupt the balance of the intestinal microflora.

In rabbits, the caecal microflora and the fermentation processes taking place in the caecum play a key role in digestion. In addition to other aetiological factors, disrupted balance of the gut flora (dysbiosis) may directly or indirectly contribute to the development of digestive disturbances or diseases. Digestive diseases may result in as high as 30–50% mortality in a rabbit herd, and the recovered animals markedly reduces performance (Lelkes and Chang, 1987).

The rearing losses occurring during meat rabbit production are largely attributable to diseases of the digestive tract and the mortality resulting from that (Gidenne and Fortune-Lamothe, 2002). About 25% of these losses occur in the period around weaning, i.e. between 18 and 50 days of age.

Disruption of eubiosis may result in enteritis, which is a collective term, as the clinical signs caused by different pathogens are very similar, with diarrhoea being their common feature. Digestive diseases in rabbits are often caused by pathogenic microorganisms and environmental factors, mainly feed change and inadequate keeping conditions such as poor hygiene and stress (Lelkes, 1986). Therefore, in rabbits nonspecific enteropathy is applied as a term for this process (Peeters et al., 1988), which is characteristically of multifactorial aetiology (Klis and Jansman, 2002).

The administration of antimicrobial agents is the most common practice to control digestive diseases, especially in farm animal productions (Chevance and Moulin, 2009).

2.3.1. Some consequences of antibiotic use

Regarding antibiotic use, a distinction should be made between the previously practiced subtherapeutic antibiotic application and the therapeutic use of antibiotics, which is permitted also at present.

Earlier, by feeding antibiotics at a subtherapeutic dose a marked (10–25%) growth promotion and good production stability were achieved. According to the supposition of Szigeti (2003) the feeding of antibiotics markedly reduces the number of pathogenic bacteria and the relative ratio of the satellite flora in the digestive tract, while the number of useful commensal bacteria is only slightly or not at all reduced, and their proportion may even increase.

Research so far indicates that continuous antibiotic use (e.g. avoparcin and virginamycin) may result in the selection of antibiotic-resistant bacterial strains and their enrichment in the environment, which can cause infections in humans and/or animals (Chapin et al., 2005; Rönner et al., 2004; Fallon et al., 2004). According to Knopp et al. (2010), soil-dwelling bacteria possess a continually increasing number of antibiotic resistance genes. This increases the risk of antibiotic resistance genes being transferred from innocuous bacteria to disease-causing ones (e.g. methicillin-resistant *Staphylococcus aureus*).

Using denaturing gel electrophoresis, Abecia et al. (2004) studied the effects by certain antibiotics added to the feed on the caecal ecosystem, and found that chlortetracycline and tiamulin caused major changes in the caecal microflora.

Digestive disorders and especially epizootic rabbit enteropathy (ERE) adversely affected the health status of rabbit farms and increased the preventive use of antibiotics (Duperray et al., 2003).

One of the European Union fundamental principles is that European consumers have the right to safe food. The legislation of the European Community and the scope of the 'farm to fork' quality assurance principle includes the conditions of animal production and, thus, also the composition of feed.

The European Union (Directive 2001/82/EC of the European Parliament and of the Council) made provisions on prohibiting the use of antibiotics as growth promoters (Casewell et al., 2003), and this ban came into force on 1 January 2006. As Hungary is a member of the European Union, antibiotic supplementation of the feed of farm animals for preventive or growth promotion purposes is not permitted in Hungary either. Regulation (EC) No 470/2009 of the European Parliament and of the Council, as well as

Commission Regulation (EU) No 37/2010 specified the maximum residue limits of antibiotics permitted in foodstuffs of animal origin.

2.3.2. Possibilities for the replacement of antibiotics

The restrictions of antibiotic use in recent years have made it an urgent task to replace antibiotics with other products having probiotic or antimicrobial effects. According to Szigeti (2003), long-term efforts will be needed before antibiotics increasing both the safety of production and the production yields can be replaced with full-value alternatives.

Probiotics and prebiotics can change the composition of the intestinal microbiota and stabilise the health status of animals (Williams and Newbold, 1996; Bosi et al., 2001; Medina et al., 2002).

2.3.2.1. Probiotics

Probiotics are feed additives containing viable microorganisms, which ensure the healthy functioning of the digestive tract that can modulate the activities of the digestive microbiota in order to improve the health or performance of the host (Marteau et al., 2002). They do not destroy pathogenic bacteria, but provides a barrier function against them by preventing their development and colonisation (Maertens et al., 1992).

According to Abbott (2004), the possible mechanisms of action of probiotics are as follow: they increase the metabolic activity of the intestine, modify the composition of the intestinal microbiota through the competitive exclusion of pathogenic bacteria, modify the structure and function of the intestinal epithelium, and stimulate the immune system.

Probiotic microorganisms have been used in animal feed since the end of the 1980s and their application as feed additives is started to be regulated in 1993 (Council Directive 70/524/EEC) in animal nutrition. After

a transition period, which ended in the year 2000, each microbial strain has to be assessed by the EU committees and authorised by a Commission Regulation.

As probiotic microorganisms usually do not colonise the digestive tract permanently, probiotic supplementation of animal feeds should be provided on a continuous basis (Szigeti, 2003).

According to Fuller (1989), probiotics increase the absorption of minerals and reduce the excretion of ammonia and urea into the environment. On the other hand the biological effects of probiotics are highly dependent on the microorganism strain used, their ability to maintain the metabolic activity in the digestive environment and their cellular concentration.

Probiotic germs are microorganisms usually having enhanced ability for bioregulation, which belong to bacteria (e.g. to the genera *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus* and *Bacillus*) or fungi (e.g. to the genera *Saccharomyces* and *Arxyozyma*). In rabbit production, some commercial probiotic preparations mainly containing lactobacilli (Probiocin, Benebac, Probios) or bacilli (Toyocerin[®], BioPlus 2B[®]) have already been used. Lactic acid producing bacteria (LAB, e.g. *S. faecium*, *L. acidophilus*) are able to resist the acidic environment, but lactic acid has bactericidal function for other bacteria such as *E. coli* (Gippert et al., 1992). However, in the rabbit digestive tract, lactobacilli do not represent a large part of the microbial flora (Yu and Tsen, 1993; Linaje et al., 2004).

The advantage of spore-forming bacteria (like *B. licheniformis* and *B. subtilis*) is that they are able to survive the pelletisation process (Bosch, 1995). Supplementation of the diet of young rabbits with such bacteria was found advantageous, as it reduced the mortality and sanitary risk during the fattening period under summer conditions (Kustos et al., 2004).

A probiotic product containing the mixture of *Streptococcus faecium*, *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* (1.25 g/litre of drinking water) reduced the incidence of enteritis by 50% (Hollister et al., 1989). When supplemented to the diet at the rate of 0.5, 1.0 and 1.5 g/kg of feed), the same product had no influence on the growth performance of rabbits (Ismail et al., 2004).

When mixed into the diet at a rate of 0.5 g/kg, a probiotic product containing *Lactobacillus acidophilus* bacteria ($\log_{10}$ 8.9 CFU/g) improved the feed conversion ratio, increased the number of cellulolytic bacteria and decreased the ammonia content of the caecum (Amber et al., 2004).

Enterococci are part of the autochthonous gut flora of man and animals and the efficacy of their probiotic application was already demonstrated by many authors (Franz et al., 2003; Laukova et al., 2003; Simonova et al., 2008). Enterococci colonise the digestive tract of rabbits and belong to the lactic acid bacteria, which are often show probiotic character (Salminen et al., 1998). Nevertheless, this genus is not dominant member of intestinal tract. Laukova et al. (2006) reported sufficient survival and antimicrobial effects of an enterocin A producing *Enterococcus faecium* strain in rabbits.

Bacillus subtilis did not improve the growth or the health status of growing rabbits (Cristofalo et al., 1980; Lambertini et al., 1990) but combined with *B. licheniformis*, both the growth rate and the feed conversion ratio were enhanced (Zoccarato et al., 1995; Bonanno et al., 1999).

Dietary supplementation with a combination of *B. subtilis* and *B. licheniformis* ($\log_{10}$ 5.1 CFU/g) at 400 mg/kg diet had no effect on weight gain or feed conversion ratio, but decreased the mortality and morbidity risk (Kustos et al., 2004).

In their experiment conducted with does and their litters, Maertens et al. (1992) supplemented the diet with a substance containing *Bacillus* (strain CIP 5832) spores ($\log_{10}$ 6 CFU/g), and showed an increase in weaning weights. According to Vörös and Gaál (1992), the daily weight gain, feed conversion or mortality rate of rabbits were not modified by dietary supplementation with the same strain (0.01%).

Supposed mechanism of action for live *Saccharomyces cerevisiae* is that yeast cells survive the process of digestion and during their passage through the digestive tract they bind onto the surface of *E. coli* bacteria (Maertens and De Groote, 1992). The yeast used as feed additive had a favourable effect on the growth and health status of rabbits (Maertens and Ducatelle, 1996). For the same reason, mannan oligosaccharides (MOS) derived from the outer cell wall of *S. cerevisiae* could be used as a prebiotic. In contrast, according to the results of Jerome et al. (1996), the growth performance of rabbits was not improved by live yeast ($\log_{10}$ 6 CFU/g feed), oxytetracycline (200 ppm) or both combined, respectively. Similarly, diet supplementation with 10 g/kg *S. cerevisiae* ($\log_{10}$ 7 CFU/g DM) did not greatly modify the caecal biotope and microbiota of caecal content in rabbits (Kimse et al., 2012).

When we started our studies according to the EEC (2001), on the territory of the European Union only two probiotics were authorised for use in rabbit diets: *Bacillus cereus* var. *toyoi* (Toyocerin[®], EC no. E-1701) and *Saccharomyces cerevisiae* (Biosaf[®], EC no. E-1702) (Pinheiro et al., 2007).

2.3.2.1.1. The application of *Bacillus cereus*

Bacillus cereus is a rod-shaped, Gram-positive bacterium 3–5 μm × 1 μm in size. It forms an endospore of ellipsoidal shape and belongs to the

Bacillaceae family. According to its relationship with oxygen, it is a facultative anaerobic bacterium (Adams and Moss, 2000).

The temperature optimum of growth of *B. cereus* is 28–35 °C, the minimum pH value for activity is 5–6, and the minimum water activity value is 0.95. This bacterium can be found in the soil and water, on the vegetation and it commonly occurs among the transient intestinal microbes in humans (Williams et al., 2009).

Some *B. cereus* strains can produce an enterotoxin that may cause vomiting and diarrhoea (Granum and Lund, 1997). In an experiment conducted by Williams et al. (2009), isolated ileal loops were injected with Toyocerin[®] (feed additive containing *B. cerus*) and *B. toyoi* preparations containing up to $\log_{10}$ 10 spores/ml or concentrated up to 100-fold and observed the signs of enterotoxicity for up to 25 h. No adverse effects or signs of enterotoxicity were reported in the ileal loops of the rabbit.

European Commission authorized the use of the *Bacillus cereus* var. *toyoi* (NCIMB 40112/CNCM I-1012) product belonging to the group of microorganisms as a feed additive for broiler chickens and fattening rabbits. Subsequently, this feed additive product was included in the Community register of feed additives (2006), in harmony with Article 10(1) of Regulation (EC) No 1831/2003 of the European Parliament and of the Council.

The product designated Toyocerin[®] contains viable *Bacillus cereus* var. *toyoi* (NCIMB 40112/CNCM I-1012) spores in a minimum concentration of $\log_{10}$ 10 CFU/g. This microbe is not a genetically modified organism.

Most of the experiments performed with *B. cereus*-containing products in several animal species demonstrated an increase in *Lactobacillus*

counts in the small intestine and a decrease in coliform counts in the large intestine (SCAN, 2001).

In piglets, Toyocerin[®] treatment resulted in a significant increase in body weight gain as compared to the control group (Taras et al., 2005; Schierack et al., 2009). In a long-term feeding trial in cattle, Toyocerin[®] did not cause any adverse effects when administered at $\log_{10}$ 9.3 spores/kg of body weight/day for 18 months. Increasing Toyocerin[®] level ($\log_{10}$ 5, 6 and 6.7 *Bacillus toyoi* spores/g feed) caused a significant reduction in *E. coli* numbers, resulted in the complete prevention of diarrhoea and provided a favourable effect on live weight gain in rabbits (Hattori et al., 1984).

According to Nicodemus et al. (2004), the inclusion of 200 ppm Toyocerin[®] in the diet of lactating rabbit does improved reproduction (numerical productivity increased by 19%), and litter weight (by 7,6% at 25 day weaning age). Supplementation of the diet with 200 ppm Toyocerin[®] (concentration: $\log_{10}$ 9 *Bacillus cereus* var. *toyoi* spores/g) significantly increased the weight gain, improved the feed conversion and reduced morbidity. No significant effect was observed with a higher inclusion rate in the diet (1000 ppm) (Trocino et al., 2005).

Toyocerin[®] increased feed intake by does with litters between day 18 of lactation and weaning. The weight of the kits at weaning was higher (by 4,9% and 5,6%) in the probiotic groups (Toyocerin 200 and T 1000) than in the control group. The increase in fertility could be due to the better health status of females. The probiotic product reduced the mortality of kits. The addition of 0.2 g Toyocerin/kg feed had effects similar to those obtained with an inclusion level of 1 g/kg (Pinheiro et al. (2007).

Dietary supplementation with 1000 ppm of Toyocerin[®] did not affect the growth and the feed conversion ratio, but significantly reduced the

mortality and the sanitary risk index of rabbits during the fattening period (Pascual et al., 2008).

Analysis of the complete genome sequence showed that the strain harbours all of the genes coding for non-haemolytic and haemolytic enterotoxins. Toyocerin strain has the capacity to elaborate functional toxins (EFSA, 2012).

2.3.2.2. Prebiotics

Prebiotics are food ingredients that selectively stimulate the growth and metabolic activity of one or a limited number of bacterial species of the intestinal microflora (usually of species belonging to the genus *Lactobacillus* and/or *Bifidus*), thus improving host health and well-being (Cummings et al., 2001; Roberfroid, 2001).

According to Quigley (2008), the prebiotics are oligosaccharides that are not absorbed or digested in the small intestine of animals, but fermented by the intestinal microflora. They increase the number of beneficial micro-organisms, while repressing the harmful bacteria.

According to the definition proposed by Gibson et al. (2004, 2005), prebiotics are nutrients selectively digestible in the colon, which pass the small intestine in chemically unchanged form and stimulate the growth of beneficial microorganisms in the colon.

Prebiotic oligosaccharides are defined as non-digestible food ingredients that stimulate selectively the growth and/or activity of potentially health-enhancing intestinal bacteria (Flickinger et al., 2003)

Prebiotics primarily stimulate the growth of bifidobacteria, thus supporting the prevention and elimination of intestinal infections of exogenous and endogenous origin (Szigeti, 2003).

According to Roberfroid (2000), the use of prebiotics reduces the risk of osteoporosis, type II diabetes, insulin resistance and associated obesity in humans. Prebiotics may also have importance in tumour prevention. According to Reddy et al. (1997), the number of aberrant crypts induced by colonic carcinogens (e.g. azoxymethane and dimethylhydrazine) was significantly lower in rats fed inulin. In some cases, prebiotics were found to stimulate the degradation of endogenous carcinogens (e.g. sialomucin) by the intestinal microflora (Cassidy et al., 1990). Prebiotics depressed the growth of tumours in rodents (Taper et al., 1997). According to the results of a study, β-(2-1)-fructans stimulate the apoptosis of colonic epithelial cells, which is a significant anti-cancer effect (Hughes and Rowland, 2001).

Of the prebiotics, oligosaccharides (mannan, fructose, glucose oligosaccharides) already have a wide range of applications in animal production as well.

The addition of mannan oligosaccharide (MOS) to the diets resulted in better intestinal integrity and had a protective effect against common pathogens (Guedes et al., 2009). It is possible to bind the mannose receptors of some pathogenic bacteria (e.g. *E. coli* and *Salmonella enteritidis*) in order to prevent their attachment to the intestinal mucosa (Spring et al., 2000). At a concentration of 1 g/kg, MOS induced a significant reduction in the mortality rate in rabbits under critical conditions involving an episode of ERE. Mannan oligosaccharide (1 g/kg diet) could be used as an alternative to antibiotics during rabbit growth, and led to positive effects on body weight, nutrient digestibility and the fermentative activity of the caecal microbial population (Bovera et al., 2010).

The performance parameters of growing rabbits fed gluco-oligosaccharide- (GOS) supplemented diets did not significantly differ from those fed a diet without GOS supplementation (Gidenne, 1995; Peeters et al.,

1992). However, Gidenne (1995) observed a significant increase in morbidity (30%) and mortality (24%) caused by GOS supplementation. The morbidity was 18% and mortality was 15% during the fatterining period in the control group.

According to Morisse et al. (1993), a fructose-oligosaccharide- (FOS) supplemented (0.25%) diet of rabbits decreased the number of *E. coli* O103, caecal pH and NH_3, while it increased SCFA production and improved the liveweight. Fructose oligosaccharide added at a level of 0.24% to rabbit diet improved the weight gain, and increased the pH of caecal content (Aguiler et al., 1996). On the other hand, FOS (at 0.34% of diet) had no effect on growth performance (Lebas, 1996) and SCFA production or on microbes causing diarrhoea (FOS at 0.36% of diet, Maurao et al., 2004).

2.3.2.2.1. Inulin

Inulin is a fibrous substance commonly occurring in plants; a fructose polymer. It was first isolated by Rose in 1804. It can be found in the rhizome and subterranean parts of plants belonging to the families Liliaceae, Compositae, Amarillidaceae and Gramineae (e.g. dahlia, dandelion, artichoke, squill and beans; Szabó and Szabó, 2003). In human nutrition, the commonest sources of inulin are wheat, onion, banana, garlic and leek (Van Loo et al., 1997). The source for the large-volume industrial production of inulin is the chicory (*Cichorium intybus*). Native inulin is treated with inulinase enzyme which breaks it down into short-chain fructans. These are mainly oligofructans with an average polymerisation degree of 5 monosaccharide unites. Long-chain fructans are produced by a physical separation technology, as it undergoes colloidal dissolution in warm water; then, when it is cooled down, its main mass is precipitated again in the form of fine granules. Because of its β(2-1) bond, this structure resists digestion in

the upper segments of the digestive tract but it undergoes fermentation in the large intestine (Nines, 1999).

Inulin easily undergoes acidic hydrolysis, which takes place already as a result of prolonged boiling in diluted acetic acid or even in water. The acidic hydrolysis of inulin produces a large amount of D-fructose. In addition, D-glucose is also produced, in a proportion of approx. 6%. During methylation, each hexose component takes up three methyl groups, and inulin is converted into trimethylinulin. The acidic hydrolysis of trimethylinulin yields 3,4,6-trimethyl-D-fructose (91%), 1,3,4,6-tetramethyl-D-fructose (3.2%), tetramethyl-D-glucose (2.2%) and trimethyl-D-glucose (3.6%). These products can be separated quantitatively by extraction and chromatographic methods. The products of hydrolysis do not include dimethyl derivatives, and thus the possibility of branching is excluded. According to enzyme cleavage experiments, not only fructose but also sucrose (saccharose) is produced during the hydrolysis of inulin (Bruckner, 1961).

Inulin is a polydisperse molecule, in which the fructose units constituting the linear branch are linked to one another by β (2-1) bonds, and a terminal glucose molecule is linked to the end of the chain with an α (1-2) bond. The chemical formula of pure inulin is $(C_6H_{10}O_5)n$. The molecular weight of inulin is 6000 approximately.

Studies have shown that approx. 85–90% of ingested inulin (degree of polymerisation: 10–60) reaches the colon (Bach and Hessov, 1995).

Inulin is easily fermentable in the large intestine and is practically undetectable in the faeces (Molis et al., 1996). According to *in vivo* human studies, the fermentation of inulin selectively stimulates the growth of bifidobacteria (Madsen, 2001). The mechanism of selectivity involves general factors including a reduction of colonic pH and the formation of

metabolites inhibiting the growth of some bacteria while stimulating the growth of probiotic bacteria and exerting an antibiotic effect (Roberfroid, 2001).

Numerous medical studies have demonstrated the bifidogenic effect of inulin-type fructans in humans (Roberfroid, 1998; Van Loo et al., 1999). In these studies, the increase in the numbers of bacteria was measured in the stool of human volunteers whose diet was supplemented with different levels of prebiotics. Despite the fact that after inoculation of the stool with prebiotics the number of bifidobacteria changed, the results were inconsistent and the dose-effect curve was inconclusive (Roberfroid, 1998). According to Cummings et al. (2001), further studies are needed to determine the duration of action of prebiotics before the use of fructo-oligosaccharides as protective nutrients can be considered.

Roberfroid et al. (1998) as well as Roberfroid and Delzennem (2000) obtained sufficient evidence that inulin-type fructans can be used as prebiotics owing to their chemical structure.

Increasing levels (3%, 6% and 9%) of chicory inulin in the diet reduced the levels of enterobacteria and *Enterococcus* spp. in the descending colon and the rectum of pigs before slaughtering. The number of lactic acid producing bacteria (LAB) was not affected by the dietary treatments (Kjos et al., 2010). According to Yasuda et al. (2006) the supplemental dietary inulin was mainly degraded in their caecum in young pigs.

3. MATERIAL AND METHODS

Four experiments were conducted to answer the questions of objectives. All research protocols were reviewed by the Animal Use and Care Administrative Advisory Committee and approved by the Agricultural Administrative Authority (Protocol No. 00618/007/SOM/2003).

Besides investigating different parameters of growth and digestion, depending on the aim of the respective experiment, composition of the caecal microbiota and its fermentation activity were examined in each experiment.

Most of data have been published. The reference of the relevant publication is indicated in each chapter.

3.1. Effect of different weaning ages on production and digestive tract (published in ANIMAL 6:(6) pp. 894-901., 2012)

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

3.1.1. Experimental animals, housing and nutrition

Pannon White does and their kits were housed in flat-deck cages (85x55cm), whereas after weaning the growing rabbits were housed in two-level wire mesh cages (two kits per cage, 84 cages per treatment) in a closed building. Average temperature ranged from 21°C to 29°C, the light was on between 05:00 and 21:00h, and the farm had overpressure air ventilation.

A total of 504 rabbits were used in the experiment. One-day-old kits of average birth weight were distributed into litters of eight, and these litters were randomly divided into three groups of 21 (i.e. 168 animals per group) according to weaning age – rabbits weaned at the age of 21 (W21), 28 (W28) or 35 (W35) days. From 3 days before kindling up to weaning, the does were fed a pelleted non-medicated basal diet, which were formulated according to the recommendation made by De Blas and Mateos (2010) for rabbit (Table 1).

Young rabbits were allowed to consume the same diet, *ad libitum,* besides their mother's milk before weaning and then after weaning, up to the end of the experiment at 42 days of age.

Milk intake of the litter was determined weekly by weighing does before and after nursing. Feed consumption of the litter was measured by caging young rabbits separately from the mothers from 10 days of age. Body weight (BW) was measured weekly. Weight gain and feed conversion (g intake/g gain) were calculated.

Table 1: Ingredients, chemical composition and nutrients of the diet

Ingredients	%
Barley meal	5.0
Wheat bran	20.0
Dehydrated alfalfa meal	37.0
Soybean oil	2.0
Sunflower meal, 36% CP	10.0
Skimmed milk powder	2.0
Beet Pulp Dried	10.9
Beet molasses	2.0
Dried apple	8.9
Calcium diphosphate	0.5
Vitamin and minerals mixture*	0.5
Limestone	0.5
Salt	0.5
DL-methionine	0.1
HCl-lysine	0.1
Chemical composition and nutrients	**%**
DE (MJ/kg)	9.7
Crude protein	15.9
Ether extract	4.2
Fibre	18.8
Ash	7.8
NDF	31.6

* Premix provided per kg of diet: 11000 IU vitamin A, 2000 IU vitamin D_3, 2.5 mg vitamin B_1, 4 mg vitamin B_2, 1.25 mg vitamin K, 15 mg niacin, 0.3 mg folic acid, 600 mg choline, 3 mg Cu, 50 mg Fe, 15 mg Zn, 60 mg Mn, 0.5 mg I, 0.5 mg Co, 0.5 mg Co, 0.5 mg lysine and 0.5 mg methionin.

3.1.2. Samplings

At 14, 21, 28, 35 and 42 days of age, six healthy animals from each group (one animal per cage) were randomly selected and slaughtered at 14:00h. The digestive tract was removed immediately and stomach, small intestine and caecum were dissected. The quantity of the fresh gastric, small intestinal and caecal contents was measured and their pH values were determined using a pH meter (OP-110, Radelkis, Hungary).

One gram of caecal digesta was used immediately after sampling for microbiological culture, and anaerobic conditions were ensured by the use of carbon dioxide. The rest of the caecal content was weighed, frozen and stored at -80 ^{o}C until short chain fatty acid (SCFA) analyses.

The weight of the liver, heart, both kidneys and lung, as well as of the empty stomach, small intestine and caecum was measured. Relative weights were calculated; the weight of the liver, heart, kidney and lung was expressed in percentage of BW, and the relative weight of the empty stomach, small intestine and caecum was expressed as a percentage of the whole weight of the gastrointestinal (GI) organs (stomach, small intestine, caecum and colon).

3.2. Effect of *Bacillus cereus* var. *toyoi* (Toyocerin®) on caecal microflora and fermentation (published in MAGYAR ÁLLATORVOSOK LAPJA 130:(2) pp. 87-95., 2008)

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. It was also examined if there was any difference in the effect if kits consume the does' supplemented diet and so get the probiotic already before weaning.

3.2.1. Experimental animals, housing and nutrition

Pannon White, repeatedly pregnant does (randomly separated into groups) and their litters were included in this trial. One group of rabbits (Group T) was fed a diet containing 0.05% Toyocerin (200 ppm, $\log_{10}$ 5.3 *Bacillus cereus* var. *toyoi* spores /g feed, Asahi Vet. S. A., Barcelona, Spain). The dose of supplementation was selected considering the results of Trocino

et al. (2005), who found that the dose of $\log_{10}$ 5.3 *B. cereus* spores /g diet slightly decreased the digestive problems, while no significant effect was observed with a higher inclusion rate. The other group of rabbits (Group C) received an antibiotic-free diet with the same chemical composition. The composition of the diet is described in Table 2.

Table 2: Ingredients, chemical composition and nutrients of the diet

Ingredients	%
Dehydrated alfalfa meal, 16% CP	47.0
Wheat bran	15.1 **
Barley meal	14.0
Dried beet pulp	7.5
Soybean meal, 48% CP	3.0
Sunflower meal, 35% CP	9.0
Soybean oil	1.0
Cane molasses	1.4
Calcium carbonate	0.1
Dicalcium phosphate	0.9
Sodium chloride	0.4
L-lysine	0.1
DL-methionine	0.1
Vitamin-Mineral premix*	0.5
Chemical composition and nutrients	**%**
DE (MJ/kg)	10.236
Dry matter	89.900
Crude protein	15.993
Crude fat	4.246
Crude fiber	16.057
Ca	1.301
P	0.597
Na	0.197
Mg	0.009

* Premix provided per kg of diet: 10500 mg vitamin A, 958.15 mg vitamin D_3, 20.625 mg vitamin E, 1.625 mg vitamin K_3, 0.985 mg vitamin B_1, 134.51 mg Fe, 48.72 mg Mn, 9.898 mg Cu, 81.46 mg Zn, 0.002 mg Se, 0.968 mg Co, 3.195 mg I, 0.753 % Lys, 0.338% Met, 0.584% Met+Cys, 0.607% Thr, 0.208% Try,
** Diet of Group T contained 15.05% Wheat bran and 0.05% Toyocerin.

The does and their kits were housed in flat-deck cages (85x55cm), whereas after weaning the growing rabbits were housed in two-level wire mesh cages (two kits per cage, 84 cages per treatment) in a closed building. Average temperature ranged from $21^{o}C$ to $29^{o}C$, the light was on between 05:00 and 21:00h. 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 (CC, n=37 and TT, n=46), while the diet of the other two groups was changed (CT, n=38 and TC, n=44) as shown in Table 3.

Table 3: Experimental groups

Groups	Diet of does and kits		Diet of growing rabbits	
	Basic diet	Toyocerin	Basic diet	Toyocerin
CC	+	-	+	-
CT	+	-	+	200 ppm
TC	+	200 ppm	+	-
TT	+	200 ppm	+	200 ppm

C = control, T = Toyocerin, ppm = part per million

Data of morbidity and mortality were registered and „Health Risk Index" (HRI) was calculated as the sum of morbidity and mortality (Bennegadi et al., 2000). Body weight (g/rabbit) and feed consumption (g/litter/week) were measured from the 3rd week of age.

3.2.2. Samplings

At the age of 21, 28 and 35 days, six healthy animals from each group were euthanised by CO_2 gas at 14:00h. Body weight was measured after exsanguination. The digestive tract was removed immediately and the caecum was separated. The pH of the gastric and caecal content, composition and SCFA production of the caecal microbiota was measured.

3.3. **Effect of inulin supplementation and age on growth performances and digestion parameters in weaned rabbits (published in WORLD RABBIT SCIENCE 18:(3) pp. 121-129., 2010)**

The aim of the present experiments was to study the effect of age and supplementation of the diet with inulin on growth performance and certain variables of digestion, especially the caecal ecosystem and the fermentation in weaned rabbits.

3.3.1. Experimental animals, housing and nutrition

Twenty four Pannon White does and their kits were housed in flat-deck cages (85x55cm) in a closed building, with 16 light hours per day. After weaning (28 day of age) rabbits were caged in pairs (30 cages/treatment, 2 brothers/cage and 16 rabbit/m^2) in wire mesh cages until slaughter at 42 d of age. Average temperature ranged from16 to 18°C, lighting cycle was 16 h light: 8 h dark. According to the diet the litters were randomly allocated into three groups (C, M and I) at 21 d of lactation (8 litters/treatment and an average of 8 rabbits/litter).

One of the groups was fed a control diet (C) without antibiotics and inulin supplementation (containing 40.7% neutral detergent fiber and 15.1% crude protein). Rabbits of a second group (M) were fed the diet C supplemented with antibiotics (500 mg/kg oxytetracycline and 50 mg/kg tiamutin). Animals of a third group (I) were fed a diet supplemented with 4% inluin (Frutafit, HD, Brenntag, Budapest) for the barley in C diet. All diets contained Clinacox anticoccidial feed additive (0.5% diclazuril). Frutafit contained 93.2% inulin (degree of polymerization = 12), 2.9%

fructose and 3.3% saccharose. The starch content of C and M diets was 14 and 16%, respectively, but 11% in diet I. Sugar content of diets C and M was lower (7%) than in diet I (10%). The ingredients and chemical composition of the diets are shown in Table 4.

In the experimental period (between 28 and 42 d of age) body weight was measured twice a week and feed consumption was recorded weekly. Mortality was checked daily, while morbidity was assessed weekly through an individual control of all clinical signs of digestive troubles (transitory diarrhoea, presence of mucus in excreta, abnormal intake behaviour).

3.3.2. Samplings

At 28, 35 and 42 d of age, 6 healthy animals from each group (1 animal/cage) were randomly selected and slaughtered at 11:00 a.m. The digestive tract was removed immediately and the caecum was dissected. Caecal content was homogenized at room temperature. One gram of caecal digesta was used immediately after sampling for microbiological determination and anaerobic condition was provided by carbon dioxide. The rest of the caecal content was weighed, frozen and stored at -80°C.

From the frozen samples the chemical composition, fibrolytic activity, SCFA concentrations were determined. Molecular biological technics were used to get more information about bacterial community and their number in caecal content.

Table 4: Ingredients, chemical composition and nutrients of experimental diets (%)

Ingredients	Experimental diets (Groups)		
	Control	Medicated	Inulin
Barley	12.0	12.0	8.0
Soybean meal, 46% CP	3.0	3.0	3.0
Sunflower meal, 37% CP	15.0	15.0	15.0
Alfalfa meal, 19% CP	34.0	34.0	34.0
Sugar beat pulp	12.0	12.0	12.0
Molasses	1.0	1.0	1.0
Sunflower oil	1.0	1.0	1.0
Wheat bran	18.0	18.0	18.0
Limestone	0.1	0.1	0.1
Monocalcium phosphate	1.7	1.7	1.7
NaCl	0.4	0.4	0.4
Zeolite universal	1.2	1.1	1.2
Rabbit 0.5% Clinacox[1]	0.5	0.5	0.5
Premix[2]	0.2	0.2	0.2
Inulin	-	-	4.0
Oxytetracycline 50%	-	0.1	-
Tiamulin 10%	-	0.1	-
Chemical composition and nutrients			
Digestible energy (MJ/kg)	10.7	10.7	10.1
Dry matter	91.6	91.5	91.8
Crude protein	15.1	14.3	15.0
Crude fat	2.6	2.1	2.5
Crude fibre	18.7	18.7	18.7
Ashes	7.8	7.7	7.9
Nitrogen free extractable matter	47.4	48.7	47.8
Neutral detergent fibre	40.7	40.4	39.3
Acid detergent fibre	22.6	22.0	22.7
Acid detergent lignin	3.5	3.2	3.6
Starch	13.7	16.1	11.2
Total sugar	6.9	6.9	10.2

[1] 5g/kg diclazuril

[2] Dry matter: 93.1%, Ca: 29.86%, Fe: 8000 mg/kg, Mn: 3000 mg/kg, Cu: 1000 mg/kg, Zn: 6000 mg/kg, Se: 20 mg/kg, Co: 200 mg/kg, J: 200 mg/kg, A vitamin (E672): 2400000 IU/kg, D-3 vitamin (E671): 240000 IU/kg, α-tocopherol: 8000 IU/kg, K-3 vitamin: 200 mg/kg, B-1 vitamin: 300 mg/kg, B-2 vitamin: 1000 mg/kg, B-6 vitamin: 500 mg/kg, B-12 vitamin: 4000 mg/kg, Pantothenic acid: 2800 mg/kg, Folic acid: 100 mg/kg, Biotin: 24 mg/kg, Niacin: 10000 mg/kg, Colin chloride: 800010 mg/kg, Diclazuril: 200 mg/kg.

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

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In our previous in vivo experiment (4.3.), 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 microbiota and short chain fatty acid (SCFA) production.

3.4.1. Experimental design

In both experiments Pannon White rabbits (n=3) were fed with commercial diet. They were 10 and 12 week old in experiment 1 and 2, respectively. To obtain caecal samples the rabbits were narcotized by carbo-dioxide, and sacrificed. The caecal content was homogenized, and divided into two parts under stricktly anaerobic conditions.

Sample 1 (n=9) was control, while Sample 2 (n=9) was supplemented with 4% inulin (Frutafit HD, Brenntag-Hungaria Ltd.). Samples were placed into Anaerocult culture dishes (Merck, Darmstadt, Germany), in which the anaerobic conditions were provided with the help of an Anaerocult A (Merck, Darmstadt, Germany) gasifying bag. Subsequently, the samples were incubated in an LP 104 type thermostat (LMIM, Esztergom, Hungary) at 37°C for 6 and 12 hours, respectively. Samples taken after 0, 6 and 12 hours of incubation (n=3 at each sampling) were analysed for the composition of the microbiota and SCFA content.

3.5. Laboratory analyses

3.5.1. Determination of feeds' chemical compositions

Chemical composition of the diet was analysed following the recommendations of the Association of Official Analytical Chemists (AOAC, 2000): dry matter (930.15), crude protein (Kjeldahl method, 976.05), crude fat (920.39), ash (942.05), crude fibre (978.10) and total starch (996.11). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) content were determined according to ISO 16472:2006 and ISO 13906:2008. The total sugar content was analysed according to EC 152/2009.

3.5.2. Determination of the pH-values

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

3.5.3. Microbiological culturing technics

From one gram of caecal digesta, serial dilutions (1 g caecal sample + 9 ml diluent (0.9% NaCl)) were made immediately after sampling and used for microbiological determination.

The obligate anaerobe organisms were cultured on Schaedler's agar (Sharlan Chemie, Barcelona, Spain), the selectivity of which was increased by the addition of esculin (Merck, Darmstadt, Germany), neomycin (Merck, Darmstadt, Germany) and iron ammonium citrate (Sharlan Chemie, Barcelona, Spain). Gamma sterile Petri dishes (Biolab, Budapest) were placed into Anaerocult culture dishes (Merck, Darmstadt, Germany), in which the anaerobic conditions were ensured with the help of an "Anaerocult A" (Merck, Darmstadt, Germany) gas-producing bag. Subsequently, the

samples were incubated in an LP 104 type thermostat (LMIM, Esztergom, Hungary) at 37°C for 96 hours. Total aerobic bacteria were cultured on media supplemented with 5% calf blood. The samples were incubated at 37°C for 72 hours. *E. coli* and other coliform bacteria were cultured on a Cromocult differentiation medium (Merck, Darmstadt, Germany). The samples were incubated at 37°C, under aerobic conditions, for 24 hours. The amount of lactobacilli was measured on MRS agar (Scharlan Chemie, Barcelona, Spain) after anaerobic incubation at 37°C for 24 hours.

After the incubation time had elapsed, the colonies were counted according to standard (ISO 4833:2003) with Acolyte colony counter (Aqua-Terra Lab, Veszprem). The colony counts were expressed in $\log_{10}$ colony-forming units (CFU) related to 1 g of sample.

3.5.4. Measurement of fibrolytic activity in caecal contents

The fibrolytic activity of the caecal bacteria was analysed by measuring the activity of cellulase, xylanase and pectinase. The method described by Gidenne et al. (2002) was used with minor modification: the reducing sugars were quantified spectrofotometrically at 540 nm using dinitrosalicylic acid instead of p-hydroxybenzoic acid hydrazide. The quantity of released sugars was expressed as: μmol of reducing sugar/g DM caecal digesta/h.

3.5.5. Determination of volatile fatty acid concentrations

Approximately 3 g of caecal digesta was homogenized with 4.5 mL metaphosphoric acid (4.16%), then centrifuged at 10.000 g for 10 minutes and filtrated. The concentration of short chain fatty acids (SCFA) was measured from the supernatant fluid by gas chromatography (Shimadzu GC 2010, Japan; equipped with Nukol 30 m x 0.25 mm x 0.25 μm capillary

column - Supelco, Bellefonte, PA, USA; FID detector, 1:50 split ratio, 1 μl injected volume, helium 0.84 mL/min. Detector conditions: air 400 mL/min, hydrogen 47 mL/min, temperature: injector 250ºC, detector 250ºC, column 150ºC). For quantification 2-etil-butyrate (FLUKA Chemie GmbH, Buchs, Switzerland) was used as internal standard.

3.5.6. Molecular genetics investigations

3.5.6.1. DNA extraction

Total genomic DNA from about 0.2 g of caecal sample was extracted and purified with QIAamp® DNA Stool Mini kit (Qiagen Ltd, West Sussex, England) according to the manufacturer's istructions.

3.5.6.2. Characterisation of bacterial community from caecal content samples

The V3 region of the 16S rRNA genes was used as a bacterial diversity marker with the primers w49 and 5'-6FAM-labeled w34 (Delbes et al., 1998; Zumstein et al., 2000). PCR assays with 25 cycles of amplification was performed as described previously (Michelland et al., 2009) using Isis DNA Polymerase (MP Biomedicals, Illkirch, France).

The Capillary Electrophoresis – Single Strand Conformation Polymorphism (CE-SSCP) was performed on an ABI Prism 3100 Genetic (Applied Biosystems, Branchburg, New Jersey, USA). CE-SSCP profiles were aligned, normalized and diversity index was estimated using StatFingerprints program version 2.0 (Michelland et al., 2009) running on R version 2.8.3 (R development Core Team, 2008).

The Simpson diversity index was estimated on each CE-SSCP profile with $-\log_{10}\sum(ai)^2$ where ai is the relative area under the ith peak (Haegeman et al., 2008; Rosenzweig, 1995).

3.5.6.3. Determination of *Bacteroides* copy number using real time - PCR technology

The primers were modified from literature or designed using the software ARB (Ludwig et al., 2004) and the 16S rRNA genes database SSURef_96_SILVA_04_10_08_opt.arb generated by the SILVA project (Pruesse et al., 2007).

The matching efficiency of each primer and of the whole real-time PCR system was evaluated using OligoSpecificitySystem program version 1.0 running on R version 2.8.3 (R development Core Team, 2008). The matching efficiency was defined as the ratio of the number of matching sequences to the total number of sequences in the target database (Yu et al., 2005). The target group matching efficiencies were calculated with specific databases for total bacteria (249635 sequences), and *Bacteroides-Prevotella* (14359 sequences).

The upper group minus group matching efficiency corresponded to total Eucarya plus *Archaea*, total bacteria minus *Firmicutes* and total bacteria minus *Bacteroides-Prevotella* database, respectively for the total bacteria, *Firmicutes* and *Bacteroides-Prevotella* RT-PCR assays. The upper group minus target group databases contained 36996, 160025 and 235276 sequences, respectively for the total bacteria, *Firmicutes* and *Bacteroides-Prevotella* real-time PCR assays.

According to Smith and Osborn (2009), the design of primers and probe, amplicons size, melting temperature (Tm), percentage of G+C bases possibility of self complementary of the sequences were investigated using

Primer express software (version 2.0, Applied Biosystems, Branchburg, New Jersey, USA).

Assays were performed using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Branchburg, New Jersey, USA) using optical grade 384-well plates. Final volume was 10 µl in each well.

TaqMan RT-PCR technology was used for absolute quantification of total bacteria and *Bacteroides-Prevotella*. TaqMan reaction mixture contained 2.5 µl of 200-times diluted template DNA, a set of primer (200 nM) and of TaqMan probe (250 nM for total bacteria or 150 nM for *Bacteroides-Prevotella* respectively; and 5 µl of TaqMan® universal PCR master mix (Applied Biosystems, Branchburg, New Jersey, USA).

The PCR program consisted of 10 min at 95°C, followed by 40 cycles with 15 sec. at 95 °C, 1 min at 60 °C. A dissociation curve was added to SYBR Green assays to check the specificity of the amplification.

Standard curves were generated by amplification of the serial ten-fold dilutions of plasmid (from $\log_{10} 4$ to $\log_{10} 9$ copies number) containing the 16S rRNA genes sequence of *Ruminococcus albus* (acc: EF445158; 1489 pb), and *Prevotella bryantii* (acc: EF445235; 1490 pb) for quantification of total bacteria and *Bacteroides-Prevotella* respectively. Plasmid concentrations were measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). The copies numbers for each reaction were calculated from the standard curves.

3.6. 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 and probiotic experiments.

The statistical model of inulin experiments included diet, age and their interaction as main effects, which were studied by the following general linera model (GLM):

1.) $Y_{ijk} = \mu + T_i + A_j + TA_{ij} + e_{ijk}$

2.) $Y_{ijk} = \mu + T_i + A_j + e_{ijk}$,

where μ = mean, T_i = effect of the treatment (weaning age), A_j = effect of age, TA_{ij} = interaction of treatment and age, e_{ijk} = random error.

When treatment and age interaction was significant, it was included into the model (1.) If interaction (T*A) was not significant this element was omitted from our model (2.).

The significance of differences was tested by least significant difference (LSD) post hoc test. The experimental unit was the cage in the case of feed intake and feed conversion, but the animal for growth rate and other measurements.

When a significant ($P<0.05$) age x treatment interaction occurred, data were further subjected to two types of statistical analyses: within the same age (among the three diets) and within the same diet (among ages).

The Pearson's correlation was used to find a relationship between pH and *E. coli* (in experiment 4.3.). Mortality and morbidity of the groups was compared by chi-squared analysis.

3.6.1. Statistical analyses of the molecular genetics results

Environmental parameters, RT-PCR data and diversity index were subjected to ANOVA, and Tukey's HSD post-hoc test using dietary treatment (3 levels), days (d) of sampling (3 levels) and their interaction as fixed effects (R development Core Team, 2008).

The CE-SSCP profiles data were explored using a centered and scaled Principal Components Analysis (PCA) and tested with a fifty-fifty multivariate ANOVA (FF-MANOVA) with 10000 rotations (Langsrud, 2002; Langsrud, 2005).

FF-MANOVA was performed using dietary treatment, d of sampling and their interaction as fixed effects. When an effect was significant, an iterative Mann-Whitney test was applied on each scan of the CE-SSCP profile to identify the scan position which differed along the CE-SSCP profile. Such analyses on CE-SSCP profiles are referred in the text as analysis of the structure of the bacterial community.

The correlations between the environmental parameters, the diversity index and the number of genes copies of total bacteria and *Bacteroides-Prevotella*, were analyzed using a centred and scaled principal component analysis (PCA). The correlations between the CE-SSCP profiles of the bacterial community and the environmental parameters were tested using redundancy analysis (RDA) with 10000 Monte Carlo permutations (Legendre and Legendre, 1998). Prior to the RDA, the environmental parameters included in the model were selected using a stepwise selection. As environmental parameters were expressed in different units, they were centered and scaled prior to RDA.

4. RESULTS AND DISCUSSION

4.1. Effect of different weaning ages on production and certain parameters of the digestive tract

4.1.1. Body weight, milk and feed consumption

By the age of 35 days, the BW of animals weaned at 35 days of age increased by 14 and 10% (P<0.05) as compared to those weaned at 21 and 28 days of age, respectively, whereas by the age of 42 days it increased by 10%. So W35 rabbits were significantly heavier (940 g) than W21 (826 g) and W28 (850 g) animals on day 35. One week later, the difference between W35 (1175 g) and the other two groups (W21: 1062 g; W28: 1068 g) was more than 100 gramms (Table 5).

Table 5: Effect of weaning and age on live weight of rabbits (mean±S.D.; g)

Treatment (N)	Age (days)					Interaction
	14	21	28	35	42	
	Live weight					
W21	273±35^a	385±44^b	560±70^c	826±129^{dA}	1062±187^{eA}	NS
W28	247±49^a	346±67^b	542±68^c	850±133^{dA}	1068±185^{eA}	NS
W35	262±32^a	381±42^b	595±52^c	940±75^{dB}	1175±184^{eB}	NS

W21=weaning at 21 days; W28=weaning at 28 days; W35=weaning at 35 days
N = 168 rabbits per treatment, NS = P>0.05, S.D. = Standard deviation, Different subscripts indicate significant (P<0.05) defferences between [a, b, c, d, e] ages and [A, B] treatments

The milk consumption of rabbits weaned at 28 and 35 d of age increased by 40% in the 4th week. Thereafter it decreased by 66% between 28 and 35 d of age. The W35 animals decreased milk consumption by 44%

(P<0.05) in the period d 29 to d 35 relative to that measured in the earlier week.

Early weaned animals had 75% higher feed intake compared to W28 and W35 rabbits (P<0.05) in the 4th 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 between the treatment groups (Table 6).

Table 6: Effect of weaning and age on growth trait, milk consumption and feed consumption of rabbits (mean±S.D.)

Treatment	Age (days)				Interaction
	14 to 21	22 to 28	29 to 35	36 to 42	
Weight gain (g/day/rabbit)[1]					
W21	15.4±2.8	25.3±5.5	42.4±10.8	33.6±14.4	NS
W28	13.7±4.3	30.8±5.4	41.6±6.8	31.1±12.6	NS
W35	17.0±2.3	30.8±5.6	49.0±4.5	33.6±17.9	NS
Milk consumption (g/day/kit)[2]					
W21	30±6	-	-	-	NS
W28	27±5[a]	38±6[b]	-	-	NS
W35	26±5[a]	38±4[b]	25±5[a]	-	NS
Feed consumption (g/day/rabbit)[1]					
W21	1.1±0.6[a]	35±6[bB]	84±6[c]	89±10[c]	NS
W28	1.7±1.0[a]	19±8[bA]	79±9[c]	94±13[d]	NS
W35	1.4±0.5[a]	20±6[bA]	72±7[c]	99±13[d]	NS

W21=weaning at 21 days; W28=weaning at 28 days; W35=weaning at 35 days
[1]N=168 rabbits per treatment, [2]N=21 litters per treatment, S.D. = Standard deviation,
- = No data, NS = P>0.05, Different subscripts indicate significant (P<0.05) defferences between [a, b, c, d] ages and [A, B] treatments

According to these data, age and weaning influenced solid feed intake, whereas milk and feed consumption were in accordance with the milk production of the does, which reached the maximum between 19 and 21 days and then began to decline after the 26th day of lactation (Maertens et al., 2006). Young rabbits usually begin to eat small amounts of solid food in addition to the milk; however, their feed intake becomes significant only

approximately by 20 days of age (Gidenne and Fortun-Lamothe, 2002). The W21 rabbits consumed more pellet than those weaned at 28 or 35 days of age; however, this higher intake of solid feed was not enough to provide a growth similar to that of W35 animals. Thus, early weaning resulted in a significant reduction in growth compared with weaning at the age of 35 days. This finding is very similar to that of Cesari et al. (2007), who compared the growth performance of rabbits weaned at 25 v. 34 days of age, and also with that of Gallois et al. (2008), who reported lower growth in early (on day 21) than in traditionally (on day 35) weaned rabbits until 42 days of age. Xiccato et al. (2003) also showed that BW at 32 days of age was in a positive correlation with the weaning age (at 21, 25 and 28 days), but it became similar in rabbit weaned at different times by 56 days of age.

4.1.2. Age related development of organs

The relative weight of the liver increased by 62% between 21 and 28 days of age (Table 7); thereafter there was a decrease (by 76%) between days 35 and 42 (P<0.05). On day 35, W21 rabbits had higher relative liver weight (by 29%) as compared with W35 animals (P<0.05). Total relative weight of viscerals (heart, kidneys and lungs) measured in this experiment decreased with age (by 70% between days 14 and 42).

The weight of the GI organs was found consistent with most of the relevant weight data in the literature (Lebas and Laplace, 1972; Alus and Edwards, 1977; Piattoni et al., 1995). When the young begin to eat significant quantities of solid feed, the weight of the GI tract starts to increase (Alus and Edwards, 1977).

The relative weight of the GI tract increased by 49% between 21 and 28 days of age in W21 (P<0.05) and by 22% between days 28 and 35 in W28 rabbits (P<0.05), respectively. Early weaning and a switch to solid feed

manifested in earlier growth of the GI tract: on day 28, the relative weight of the GI tract was 19% higher in W21 rabbits than in W28 animals, whereas on day 35, W21 and W28 animals had a higher weight of GI tract, with a 12% increase as compared with W35 animals (P<0.05). Considering these laters, an interaction (P<0.05) was shown between age and treatment (weaning). However, age related changes influenced the relative weights of the stomach, small intestine and caecum within the GI tract, regardless of the weaning age.

Table 7: Effect of weaning on relative weight of viscerals and on different sections of GI organs[1] (mean±S.D.; %)

Treatment (N)	Age (days)					Interaction
	14	21	28	35	42	
	Liver/BW[2]					
W21	2.9 ± 1.0^a	2.7 ± 0.9^a	4.5 ± 1.5^{bc}	4.8 ± 0.6^{cB}	3.7 ± 0.6^b	
W28	-	-	3.9 ± 0.9	4.1 ± 0.5^{AB}	3.4 ± 0.5	NS
W35	-	-	-	3.7 ± 0.6^{dA}	3.1 ± 0.6	
	Heart+kidneys+lung/BW[2]					
W21	2.8 ± 0.6^c	2.4 ± 0.8^{ac}	2.3 ± 0.5^{ab}	2.3 ± 0.6^{ab}	1.9 ± 0.6^a	
W28	-	-	2.4 ± 0.5	2.2 ± 0.4	2.2 ± 0.5	NS
W35	-	-	-	2.1 ± 0.4	2.1 ± 0.5	
	GI/BW[2]					
W21	5.5 ± 1.2^a	6.9 ± 1.6^a	10.3 ± 2.5^{bB}	10.4 ± 1.8^{bB}	10.3 ± 1.3^b	
W28	-	-	8.6 ± 2.1^{aA}	10.5 ± 1.5^{bB}	10.4 ± 1.2^b	*
W35	-	-	-	9.4 ± 1.2^A	10.1 ± 1.2	
	Emptied stomach/empty GI[3]					
W21	33.0 ± 3.5^b	23.1 ± 2.3^a	21.5 ± 2.1^a	18.9 ± 2.0^a	18.4 ± 1.3^a	
W28	-	-	22.7 ± 2.2	20.4 ± 2.1	17.7 ± 1.2	NS
W35	-	-	-	20.4 ± 2.1	20.1 ± 1.2	
	Emptied small intestine/empty GI[3]					
W21	44.1 ± 2.8^a	38.1 ± 2.6^b	32.4 ± 2.2^b	34.4 ± 1.8^b	33.1 ± 1.6^b	
W28	-	-	33.1 ± 2.3	32.4 ± 1.6	31.3 ± 1.5	NS
W35	-	-	-	31.8 ± 1.8	30.3 ± 1.6	
	Emptied caecum/empty GI[3]					
W21	10.4 ± 1.1^a	17.3 ± 1.5^b	15.5 ± 1.3^b	17.8 ± 1.2^b	20.3 ± 1.8^b	
W28	-	-	14.8 ± 1.2^a	17.5 ± 1.3^a	23.1 ± 1.6^b	NS
W35	-	-	-	16.9 ± 1.3^a	20.6 ± 1.6^b	

GI = gastrointestinal; W21=weaning at 21 days; W28=weaning at 28 days; W35=weaning at 35 days. [1] N=6 rabbits per treatment. [2] Expressed as % of BW. [3] Expressed as % of the whole GI tract (stomach, small intestine, caecum and colon), S.D. = Standard deviation, - = No data, because before weaning the groups could be considered identical. NS = P>0.05; * = P<0.05, Different superscripts indicate significant (P<0.05) differences between [a, b, c, d] ages and [A, B] treatments.

Quite similar as the effects were demonstrated on the relative weight of the individual GI organs, age also influenced the relative weight of the contents in different GI sections (expressed as % of BW); e.g. between 14 and 42 days of age, gastric content decreased (by 50%), whereas that of the

small intestinal and caecal contents increased (2.2 and 15 times, respectively), with the latter showing an age x treatment interaction (P<0.05). Seventyeight % higher, gastric content was found in W21 animals than in W35 rabbits at 35 days of age (P<0.05). The reason of the decrease in the gastric content observed at 42 d in all groups might be a temporary lack of food intake on 41 d, attributed to a sudden rise in environmental temperature (Table 8).

Despite the results of several researchers (Piattoni et al., 1995; Gallois et al., 2008; etc.) in our experiment no significant influence in the caecal development (enlargement) was resulted by the different mechanical stimulation of the solid feed in different consumption rates. Similarly, no difference in the weight of caecum and caecal content was found by Cesari et al. (2007), when early weaning (at day 25) was compared with later weaning (on day 34). There was a reversed change in the proportion between the weight of the stomach and the caecum; i.e., before weaning (day 14) the stomach accounted for 38% of the GI tract, whereas its proportion decreased to the range of 29 to 32% by the time around weaning; however, the relative weight of the caecum increased from 12% (before weaning) to the range between 29% to 32% at 42 days of age. This is also in agreement with previous finding of Lebas and Laplace (1972) and Gallois et al. (2005), who studied the role of the different digestive organs in rabbits, assigning increasing importance to the large intestine with age and with increasing solid feed intake.

Table 8: Effect of weaning on weight of GI content in different ages (mean±S.D.; %)

Treatment (N)	14	21	28	35	42	Interaction
			Age (days)			
			Fresh gastric content/BW[1]			
W21	8.7±1.1[b]	3.6±0.8[a]	6.9±1.0[bc]	5.7±0.6[acB]	4.3±0.3[a]	
W28	-	-	5.3±0.9	4.6±0.5[AB]	4.0±0.2	NS
W35	-	-	-	3.2±0.3[A]	2.6±0.2	
			Fresh small intestinal content/BW[1]			
W21	0.6±0.1[a]	0.8±0.2[ac]	2.0±0.3[b]	1.9±0.2[bd]	1.3±0.2[cd]	
W28	-	-	1.4±0.2	1.7±0.2	1.4±0.1	NS
W35	-	-	-	1.2±0.2	1.2±0.1	
			Fresh caecal content /BW[1]			
W21	0.5±0.2[a]	2.3±0.4[b]	5.7±1.2[c]	6.0±1.2[c]	6.8±1.3[c]	
W28	-	-	5.0±0.9[a]	6.1±1.3[a]	7.8±1.1[b]	*
W35	-	-	-	4.7±0.9[a]	7.2±1.2[b]	

GI = gastrointestinal; W21=weaning at 21 days; W28=weaning at 28 days; W35=weaning at 35 days, N=6 rabbits per treatment. [1] Expressed as % of BW, S.D. = Standard deviation; NS = P>0.05; - = No data, because before weaning the groups could be considered identical. *P<0.05; Different superscripts indicate significant (P<0.05) differences between [a, b, c, d] ages and [A, B] treatments.

4.1.3. The pH values, composition of the caecal microbiota and short chain fatty acid content

The pH values in the different parts of the GI tract were within the physiological limits, in accordance with age (Gidenne and Fortun-Lamothe, 2002). No significant differences were found between groups in the pH value of either the gastric or the caecal content (data are not shown).

The pH value of the stomach content decreased from 5.7 to 3.3 (P<0.05) in W21 rabbits on 21 d, and there was a further decrease to 1.6 between 21 and 42 days in all groups (P<0.05). The pH of the small intestinal content increased from 6.8 to 7.6 and 8.4 (P<0.05) from 14 d to 21 d and 28 d, respectively, in W21 rabbits. Similar to the gastric pH, there was a decrease in caecal pH in W21 rabbits between days 14 and 21 (from 7.1 to 6.4), and then the pH decreased to 6.3 by 42 d of age. A lower pH value was

expected in the caecum because of early weaning, but the differences between groups were not significant.

Most of the caecal changes occur between 21 and 28 d of age, when solid feed intake becomes significant, depending more on age that nutrition, weaning or suckling (Gallois et al., 2008). According to the age, the count of strictly anaerobic bacteria (mainly *Bacteroides*) within the caecal ecosystem was found consistent with data reported in the literature (Gouet and Fonty, 1979; Fekete, 1989; Kovacs et al., 2002; Combes et al., 2011). These were present in high amounts ($\log_{10}$ 8 CFU/g) in the caecum, already at the age of 14 d (Table 9). Their number decreased between 21 and 42 d of age in W21 rabbits (P<0.05), which can be explained by caecotrophy starting at approximately 21 d of age (Smith, 1965). The number of coliforms and aerobic bacteria decreased from 21 d of age to up the last sampling at 42 d (P<0.05). This is in agreement with other observations according to which the presence of these species is usually suppressed by solid feed consumption (Bornside and Cohn, 1965; Gouet and Fonty, 1979). Lactobacilli are not a part of the normal ecosystem of the caecum in adult rabbits (Gidenne and Fortun-Lamothe, 2002), their number (expressed in $\log_{10}$ CFU/g digesta) was 2.5 at 14 d of age, and thereafter it decreased below 2.0 (data not shown). No significant difference occurred among groups according to weaning age (Table 9).

Table 9: Effect of weaning on the caecal digesta traits in different ages (mean±S.D.)

Treatment (N)	Age (days)					Interaction
	14	21	28	35	42	
Strictly anaerobic bacteria[1]						
W21	8.3±0.6[ab]	9.5±0.7[b]	8.1±0.6[ab]	8.2±0.4[ab]	7.8±0.4[a]	
W28	-	-	7.6±0.8	8.3±0.6	8.1±0.2	NS
W35	-	-	-	7.9±0.6	8.3±0.3	
Coliforms[1]						
W21	2.8±0.6[b]	2.4±0.8[a]	2.3±0.5[a]	2.3±0.6[a]	1.9±0.6[a]	
W28	-	-	2.4±0.5	2.2±0.4	2.2±0.5	NS
W35	-	-	-	2.1±0.4	2.1±0.5	
Total aerobic bacteria[1]						
W21	6.9±1.2[b]	5.5±1.3[a]	4.6±0.2[a]	4.8±0.4[a]	4.6±0.2[a]	
W28	-	-	4.4±0.1	4.8±0.3	4.5±0.2	NS
W35	-	-	-	4.4±0.2	4.7±0.9	

W21=weaning at 21 days; W28=weaning at 28 days; W35=weaning at 35 days
S.D. = Standard deviation; NS = P>0.05; - = No data, because before weaning the groups could be considered identical. [1] Expressed in $\log_{10}$ CFU/g digesta
Different superscripts indicate significant (P<0.05) differences between [a, b, c] ages.

The concentration of the total volatile fatty acids (tSCFA) in the caecum was related to age and weaning. In connection with higher solid feed consumption, the microbial activity was increased and SCFA was produced by caecal bacteria. The tSCFA increased with age until 28 (W21) and 35 days of age (W28 and W35), respectively (Table 10).

According to our results, there was a significant difference between ages in SCFA concentration of caecal content in W21 group. Total SCFA increased significantly from 58.7 mmol/l to 100.4 mmol/l between 21 and 28 days of age. In W21 group the change from milk to solid feed happened suddenly, which caused rapid increase in caecal concentration of tSCFA. In

W28 and W35 rabbits, the graduated adaptation for solid feed took longer time.

Early weaning resulted in significantly higher tSCFA concentrations throughout the experimental period. This is in agreement with the results of the study of Xiccato et al. (2003) in which stimulated solid feed intake (early weaning) resulted in higher tSCFA production. The proportion of acetic and butyric acid within tSCFA increased, whereas that of propionic acid decreased, resulting in a decrease of the C_3:C_4 ratio with age. Early weaning (W21) resulted in higher butyric and lower propionic acid proportion on 28 d (P<0.05). Acetic acid ratio remained significantly higher in W21 and W28 animals as compared with W35 rabbits on 35 d.

Table 10: Effect of weaning on the SCFA concentrations in caecal content in 21, 28 and 35 day old rabbit (mean±S.D.)

Treat-ment (N)	Age (days)					Inter-action
	14	21	28	35	42	
tSCFA (mmol/l)						
W21	42.9±7.4[a]	58.7±9.8[ac]	100.4±12.4[bB]	93.3±17.9[bcC]	76.6±6.4[abC]	
W28	-	-	53.2±17.2[A]	71.9±14.3[B]	61.5±5.7[B]	NS
W35	-	-	-	38.2±7.8[A]	28.3±4.5[A]	
Acetic acid (%)[3]						
W21	60.0±10.2[a]	80.5±8.4[b]	78.5±3.0[b]	78.7±3.9[bB]	77.3±2.6[bAB]	
W28	-	-	74.7±4.0	79.8±2.4[B]	82.0±3.2[B]	NS
W35	-	-	-	72.4±1.9[A]	73.5±3.3[A]	
Propionic acid (%)[3]						
W21	12.3±2.2	6.2±1.9	6.3±1.6[A]	6.6±1.8[A]	7.4±1.2[A]	
W28	-	-	7.3±0.9[B]	7.1±0.9[A]	6.5±1.2[A]	NS
W35	-	-	-	9.3±1.5[B]	8.2±1.2[B]	
Butyric acid (%)[3]						
W21	9.7±2.6[a]	11.9±3.2[ab]	15.7±2.6[bB]	13.2±2.4[abA]	13.6±1.1[abB]	
W28	-	-	13.2±2.2[A]	12.2±2.1[A]	9.9±3.0[A]	NS
W35	-	-	-	16.7±2.7[B]	15.9±3.2[B]	
C3:C$_4$						
W21	1.3±0.4[b]	0.5±0.1[a]	0.4±0.1[aA]	0.5±0.1[aA]	0.5±0.1[aA]	
W28	-	-	0.6±0.1[B]	0.6±0.1[B]	0.7±0.2[B]	*
W35	-	-	-	0.6±0.2[B]	0.5±0.1[A]	

W21=weaning at 21 days; W28=weaning at 28 days; W35=weaning at 35 days; tSCFA = total volatile fatty acids; $C_3:C_4$ =propionic to butyric acid ratio
S.D. = Standard deviation; NS = P>0.05; *P<0.05; - = No data, because before weaning the groups could be considered identical. [1] N=6 rabbits per treatment; [3]Expressed as % of the tSCFA content. Different superscripts indicate significant (P<0.05) differences between [a, b, c] ages and [A, B] treatments.

Our data were in conformity with the data of the literature (Bellier et al., 1995; Padilha et al., 1995; Gidenne et al., 2002), the proportion of

propionic acid (C_3) was higher than that of butyric acid (C_4) before weaning, but thereafter this became reversed, so that the ratio of the two fatty acids ($C_3:C_4$) decreased from >1.0 to <1.0. The increase of the butyrate concentration might be explained by an increase in some butyrate-producing bacteria. This hypothesis was supported by the results of Combes et al. (2011) who described the development of the rabbit caecum microbiota and its metabolic activities from the neonatal (day 2) until the subadult period (day 70) using 16S rRNA gene approaches coupled with capillary electrophoresis single-strand conformation polymorphism (CE-SSCP). They found that at 70 days of age, the *Firmicutes* populations remained at high levels, whereas *Bacteroides-Prevotella* decreased, resulting in higher butyrate production.

The low tSCFA content (and also the decreased acetic acid proportion) found on day 42 was the result of the temporary feed refusal on 41 d, mentioned previously (5.1.2.).

Bacteria count did not correlate to pH or the SCFA production, similar to the observations of Michelland et al. (2010), according to whom only the redox potential was negatively correlated to the bacterial diversity index, whereas pH and SCFA were not correlated to the bacterial structure.

4.2. Effect of *Bacillus cereus* var. *toyoi* (Toyocerin®) on caecal microflora and fermentation

When investigating and discussing the results it has to be taken into consideration that till the 4th week of age (before weaning) there were only two groups (C and T), so parameters of the kits reflect the supplementation of their mothers' diet. Results of the 4th week (1 day after weaning) were influenced by the increased solid feed intake, i.e. the young rabbits

consumed increasing amount of their mothers' diet from the age of about 3 weeks.

4.2.1. Growth and health status

Supplementation of the does' diet increased significantly the growth of the kits. On the 3rd week BW of T rabbits was significantly ($P<0.05$) higher (462 ± 12g) than that 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 kits. The difference between BWs among groups was detectable still on the 4th week. Kits of T does (TC, TT) had higher body weight than that of C does (CC, CT).

Examining the whole experimental period, CT rabbits reached the highest BW and their feed conversion was the best as well (2.0 g/g compared to 2.1, 2.3 and 2.5 g/g, respectively; Table 11).

Table 11: Body weight, weight gain, feed consumption, and feed conversion of rabbits fed a diet supplemented with *Bacillus cereus* var. *toyoi* (Toyocerin®) after weaning (mean ± S.D.)

Treatment	Age (days)		
	28	35	42
	Body weight (g)		
CC	610 ± 53^{ab}	993 ± 48	1269 ± 85
CT	547 ± 73^{a}	1077 ± 104	1301 ± 98
TC	717 ± 42^{b}	935 ± 68	1250 ± 92
TT	653 ± 62^{ab}	1020 ± 86	1239 ± 103
	28-42		
Treatment	Weight gain (g/day)	Feed consumption (g/day)	Feed conversion (g feed/g gain)
CC	47 ± 8^{ab}	98 ± 8	2.1 ± 0.3
CT	54 ± 5^{b}	107 ± 6	2.0 ± 0.1
TC	38 ± 6^{a}	97 ± 4	2.5 ± 0.2
TT	42 ± 5^{ab}	98 ± 4	2.3 ± 0.4

S.D. = standard deviation, C = control diet, T = Toyocerin supplemented diet, before and after weaning, [a, b]Different superscripts indicate significant ($P<0.05$) differences between treatments.

Rabbits consuming supplemented diet after weaning had significantly better health condition (Table 12). Health problems were mainly due to diarrhoea. In the caecal content of dead rabbits, high number ($\log_{10}$ 6 - 7 CFU/g) of *E. coli* was detected. The high HRI of the group CC was similar to that experienced under farm conditions where non-medicated feed is used (Trocino et al., 2005).

Table 12: Morbidity, Mortality and Health Risk Index after weaning (%)

Treatment	Age (days)	
	28-35	**36-42**
Morbidity (%)		
CC	2.7	22.6[b]
CT	0	0
TC	2.3	0.05[a]
TT	0	2.8[a]
Mortality (%)		
CC	0	12.9
CT	0	6.2
TC	4.5	8.3
TT	0	0.05
HRI* (%)		
CC	2.7	35.5
CT	0	6.2
TC	6.8	8.35
TT	0	2.85

[a, b,]Different superscripts indicate significant (P<0.05) differences between treatments.
*HRI=health risk index (the sum of morbidity and mortality)
C = control diet, T = Toyocerin supplemented diet before and after weaning

4.2.2. The pH values, composition of the caecal microbiota and short chain fatty acid content

The gastric pH value is usually about 5.0 before weaning, after starting solid feed consumption it decreases and reaches the pH value around

2.0, characteristic for the adult rabbits, after weaning (Cheeke, 1987; Fekete, 1990).

The gastric pH of T groups (TC, TT) was slightly higher than in C groups (CT, CC), however differences were not singinficant. On the 5th week of age, the gastric pH value was significantly higher in TT group than in CC group (Table 13).

The pH value of the caecum is usually about 7.0 around weaning, then decreases to around 6.0 depending on microbial activity and feeding pattern (Cheeke, 1987; Fekete, 1990). Before weaning T rabbits (TC, TT) had higher pH, however at the end of the experiment the lowest pH was measured in TT rabbits.

The higher pH in the caecum in T rabbits before weaning could presumably be due to the higher milk consumption and a lower solid feed intake as a consequence. Because less amount of fermentable substrate entered the caecum, the lower SCFA production resulted in higher pH.

Table 13: The pH of the gastric and caecal content (mean±S.D.)

Groups	Age (days)		
	21	28	35
	Gastric pH		
CC	5.7 ± 0.1	2.2 ± 0.4	2.5 ± 0.6^a
CT	-	2.2 ± 0.3	2.7 ± 0.4^a
TC	-	2.6 ± 0.3	2.6 ± 0.4^a
TT	5.5 ± 0.1	2.6 ± 0.4	3.2 ± 0.4^b
	Caecal pH		
CC	6.7 ± 0.2^a	6.1 ± 0.3	6.3 ± 0.2^{ab}
CT	-	6.3 ± 0.3	6.3 ± 0.2^{ab}
TC	-	6.4 ± 0.4	6.6 ± 0.4^a
TT	7.2 ± 0.2^b	6.3 ± 0.2	6.0 ± 0.1^b

C = control diet, T = Toyocerin supplemented diet before and after weaning, n= 6 rabbit per group, S.D. = standard deviation, [a, b, c] Different superscripts indicate significant (P<0.05) differences between treatments. - = no data because CC and TT rabbits were fed with the same diet as CT and TC, respectively, until day 28

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Similarly to our previous results (Kovacs et al., 2002), the count of total aerobic bacteria was higher before, than after weaning. On the 35th day of age, CFU number of TT group was significantly greater compared to TC group, both remaining within the physiological range.

The germ counts of anaerobic bacteria growing on the Schaedler agar in the caecal content (data not shown) were similar to those experienced in previous experiments (Kovács et al., 2002, 2003; Zomborszky-Kovács, 2000). Before weaning, the CFU number of *Bacteroidetes* was slightly higher, than it decreased, simultaneously with the improving intensity of coecotrophy. After weaning, supplementation had no significant effect on *Bacteroidetes*.

The most characteristic finding was the significant difference in the number of coliforms, being higher in C rabbits on the 21st d (Table 14). The difference disappeared by the 29th day of age, thereafter, by day 35 Toyocerin caused significantly less coliforms in groups CT and TT. For coliforms the count of $\log_{10}$ 3 - 4 bacteria/g chyme in group TC could be considered as physiological, 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 14: Composition of the caecal microbiota (mean±S.D.)

Groups	Age (days)		
	21	**28**	**35**
Total aerobic bacteria ($\log_{10}$ CFU/g chyme)			
CC	6.7 ± 0.7	4.6 ± 0.9^a	4.6 ± 0.7^{ab}
CT	-	5.6 ± 1.1^b	4.5 ± 0.4^{ab}
TC	-	4.5 ± 0.7^a	4.0 ± 0.4^a
TT	6.3 ± 0.9	4.5 ± 0.6^a	5.0 ± 0.8^b
Bacteroides ($\log_{10}$ CFU/g chyme)			
CC	10 ± 0.2	8.9 ± 0.6^{ac}	8.7 ± 0.5
CT	-	9.6 ± 0.4^b	8.7 ± 0.3
TC	-	9.5 ± 0.3^{bc}	9.1 ± 0.8
TT	9.8 ± 0.5	9.0 ± 0.5^c	8.8 ± 0.3
Coliforms ($\log_{10}$ CFU/g chyme)			
CC	5.9 ± 1.1^a	3.0 ± 0.1	5.0 ± 0.0^a
CT	-	3.0 ± 0.3	2.0 ± 0.1^b
TC	-	3.0 ± 0.2	3.3 ± 0.3^c
TT	4.3 ± 1.5^b	3.0 ± 0.1	2.0 ± 0.0^b

S.D. = standard deviation, C = control diet, T = Toyocerin supplemented diet before and after weaning, n= 6 rabbit per group, [a,b] significant difference between groups ($P<0.05$), CFU = colony forming unit, - = No data, because before weaning the groups could be considered identical.

There was no significant difference between groups in the total volatile fatty acid (tSCFA) content or the individual volatile fatty acid concentrations (acetic acid, propionic acid and butyric acid) except at 3rd weeks of age, when tSCFA concentration temporary increased in CC and CT groups (Table 15).

With correspondance to the literature (Gidenne, 1996; Kovács et al., 2002) the weight proportion of the acetic acid (within the tSCFA content) was between 75-80%. The ratio of the propionic acid was above 10% before weaning, but thereafter it decreased to 5.5-7.0%. In contrast, the percentage of butyric acid increased from 5.5-6.5% to 14.0-17.0%, so the ratio of these two fatty acids (C_3:C_4) decreased from 2.0 to 0.4-0.5.

Table 15: Volatile fatty acid production (mean± S.D.)

Groups	Age (days) 21	28	35
	Total volatile fatty acid content (mmol/kg)		
CC	74.0 ± 11.8	107.8 ± 2.3[b]	73.2 ± 8.1
CT	-	103.6 ± 4.2[b]	80.7 ± 3.3
TC	-	80.5 ± 8.2[a]	66.4 ± 4.4
TT	65.6 ± 4.5	79.9 ± 2.7[a]	74.3 ± 9.3
	Acetic acid (mol %)		
CC	79.5 ± 1.7	75.2 ± 1.9	75.5 ± 2.1
CT	-	77.4 ± 0.8	77.4 ± 0.8
TC	-	74.6 ±1.1	78.6 ± 1.2
TT	76.9 ± 1.4	75.4 ± 0.6	78.0 ± 0.9
	Propionic acid (mol %)		
CC	11.3 ± 0.8	6.3 ± 0.9	7.4 ± 1.2
CT	-	6.5 ± 0.6	5.4 ± 0.2
TC	-	6.7 ± 0.5	6.6 ± 0.4
TT	12.5 ± 1.4	6.2 ± 0.7	6.5 ± 0.6
	Butyric acid (mol %)		
CC	5.5 ± 0.9	17.9 ± 1.8	16.0 ± 1.5
CT	-	15.6 ± 0.9	16.4 ± 0.9
TC	-	17.8 ± 0.9	14.1 ± 1.0
TT	6.4 ± 0.6	17.6 ± 0.9	14.5 ± 0.4
	C3 / C4		
CC	2.1 ± 0.2	0.35 ± 0.06	0.46 ± 0.02
CT	-	0.42 ± 0.06	0.33 ± 0.03
TC	-	0.37 ± 0.08	0.46 ± 0.05
TT	1.9 ± 0.2	0.35 ± 0.10	0.44 ± 0.04

S.D. = standard deviation, n= 6 rabbit per group, C = control diet, T = Toyocerin before and after weaning, supplemented diet, [a, b, c] Different superscripts indicate significant ($P<0.05$) differences between treatments. - = no data because CC and TT rabbits were fed with the same diet as CT and TC, respectively, until day 28

4.3. Effect of inulin supplementation and age on growth performances and digestion of weaned rabbits

4.3.1. Live weight, feed intake and health status

Live body weight, feed intake and feed conversion ratio increased and growth rate decreased with age (P<0.05, Figure 1-4).

Reduced feed consumption was detected in rabbits fed inulin diet (84.5 g/day) on 6th week compare to control group (95.3 g/d) and rabbits fed medicated diet (M, 100.8 g/d).
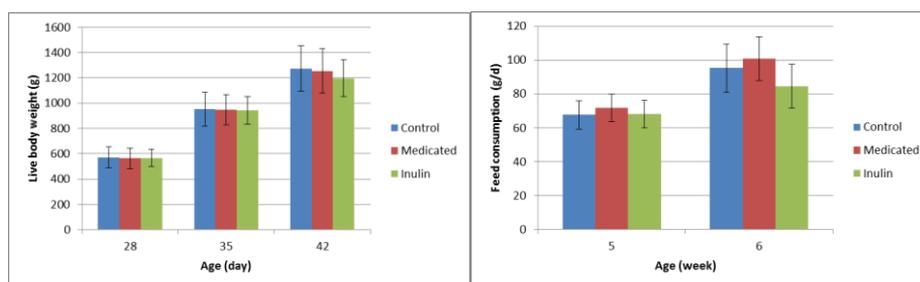

Figure 1:
Effect of age related changes and dietary treatment on live body weight of weaned rabbits
n=60 rabbits/treatment, 30 cages/treatment, residual standard deviation = 305
Figure 2:
Effect of age related changes and dietary treatment on feed consumption of weaned rabbits
(residual standard deviation = 17.8)

The reduction of feed intake (Figure 2) observed in rabbits fed inulin containing diet led to a lower daily weight gain (36.0 g/d) compared to group C and M (46.5 and 44.g/d, respectively) in the second week after weaning (Figure 3).

Growth rate on 5th week of age was not affected by type of diet, but rabbits fed I diet reduced it by 24% on 6th week of age (Figure 3), with no effect on feed conversion ratio (Table 4). Feed conversion of rabbits group I

was 2.39 g feed/g gain compared to rabbits group C (2.13 g/g) on the 6th week of age (Figure 4).
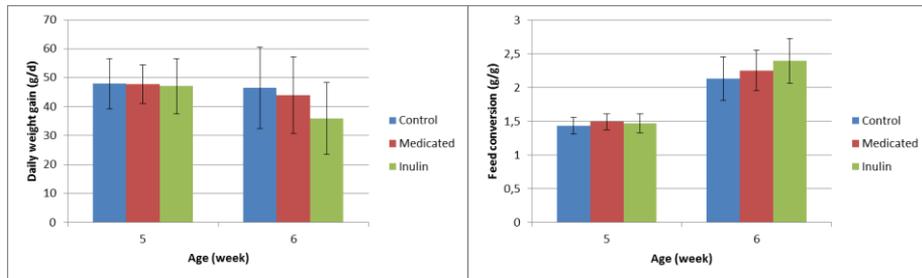
Figure 3:
Effect of age related changes and dietary treatment on daily weight gain of weaned rabbits
(residual standard deviation = 12.2),
Figure 4:
Effect of age related changes and dietary treatment on feed conversion of weaned rabbits
(residual standard deviation = 0.70), Feed conversion (g feed/g gain)

In similar experiments, supplementation of the diet with 4% inulin had no effect on the weight gain of rabbits (Volek et al., 2005). While inclusion of 4% inulin had no effect on feed intake (Volek et al., 2007), 4% inulin in a diet rich in soluble fibre (of sugar beet pulp) was found to reduce feed intake and feed conversion (Volek et al., 2005).

The inclusion of 2% inulin in the diet fed for 66 day after weaning also reduced dry matter intake (by 14%), but increased daily gain (by 16%) in rabbits (Alvarado-Loza et al., 2009). According to Maertens et al. (2004) feed consumption was decreased due to dietary inulin supplementation (2%), with no significant effect on live weight. It seems that 2-4% inulin inclusion in the diet reduces feed intake, although data from the literature are contradictory on this point.

Morbidity was higher in inulin supplemented group ($P<0.05$), and between 35-42 days of age ($P<0.05$). Symptoms of morbidity were acute

diarrhoea, low feed consumption and weakness. No effect of age and treatment on mortality was observed. In our work mortality was low and significant differences were not detected between groups.

However, and in contrast with some of the literature that found a favourable effect of dietary inulin in prevention of digestive disorders (morbidity in Volek et al., 2007, and mortality in Volek et al., 2005) inulin fed rabbits showed a higher morbidity. Lack of consistency in the results concerning the effect of prebiotics in rabbits may be explained by differences in the experimental conditions, especially those related to the hygienic status of the farm (Falcao-e-Cunha et al., 2007).

4.3.2. Effect of inulin or medication on caecal microbiota, SCFA concentration and enzyme activity

Due to inulin supplementation of the feed, xylanase activity was decreased (by 18%, $P<0.05$) compared with C and M diets (Table 16). Total SCFA concentration was not affected by diet, except in group C, where a temporary decrease by 40%, caused by the weaning, was observed on day 35 ($P<0.05$, Figure 5). This temporary change in the caecal fermentation pattern could be prevented by inulin supplementation and medication of the feed and caused a significant treatment x age interaction in tSCFA concentration.
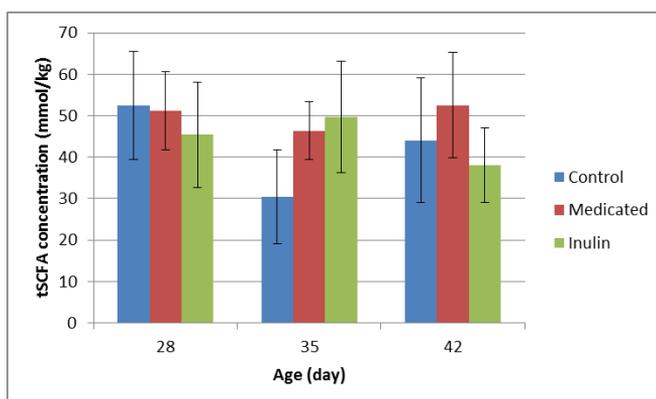
Figure 5: Effect of age related changes and dietary treatment on caecal total short chain fatty acids (tSCFA, wet content) concentrations in weaned rabbits
(residual standard deviation = 12.9 mmol/kg)

Medicated diet caused lower proportions of acetic acid than C and I diets, and higher of propionic and butyric acids compared to I diet (P≤0.05), whereas C diet showed intermediate values. Weight of caecal contents and its DM content increased with age, respectively (P<0.05), with no effect of treatments (Table 16).

Caecal pH and counts of *E. coli* and total aerobic bacteria temporarily increased and pectinase activity decreased (P < 0.050) at 35 d of age compared to 28 and 42 d of age. 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 higher at 35 d (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<0.050) at 42 d of age compared to 35 d of age, whereas these traits remained unchanged between 28 and 35 d. Propionic acid concentration decreased with age from 28 to 42 d by 23% (P<0.05).

Table 16: Effect of medication and inulin inclusion on caecal digesta traits in weanling rabbits

Experimental diets			Age (days)			RSD	P-value		
C	M	I	28	35	42		Diet	Age	Inter-action
Caecal content, % BW									
4.14	3.93	4.28	2.04[a]	4.49[b]	5.84[c]	1.99	0.632	0.001	0.566
Caecal DM content, %									
20.5	21.5	20.7	18.6[a]	20.7[b]	23.5[c]	3.0	0.393	0.003	0.685
Caecal pH									
6.48	6.40	6.57	6.23[a]	6.71[b]	6.51[ab]	0.44	0.465	0.005	0.712
E. coli[2]									
4.6	4.1	4.4	3.9[a]	4.9[b]	4.4[ab]	1.1	0.289	0.022	0.101
Strictly anaerobic bacteria[2]									
9.3	9.6	9.4	9.6[b]	9.9[b]	8.9[a]	0.7	0.374	0.000	0.096
Total aerobic bacteria[2]									
5.8	5.8	6.0	5.5[a]	6.5[b]	5.6[a]	0.7	0.548	0.000	0.948
Cellulase[3]									
78.2	72.4	70.2	69.9[a]	67.1[a]	83.7[b]	14.6	0.156	0.001	0.330
Xylanase[3]									
142[B]	139[B]	115[A]	107[a]	126[a]	163[b]	43	0.048	0.000	0.069
Pectinase[3]									
105	108	98.8	102[b]	67.6[a]	142[c]	41.1	0.603	0.000	0.346
Total SCFA (mmol/kg)									
42.3	50.1	44.4	49.7	42.2	44.9	12.9	0.136	0.166	0.044
Acetic acid (mol %)[4]									
79.9[B]	75.2[A]	81.6[B]	77.9	79.6	79.3	5.2	0.000	0.514	0.286
Propionic acid (mol %)[4]									
7.50[AB]	8.88[B]	7.15[A]	9.09[b]	7.45[ab]	6.99[a]	2.43	0.044	0.012	0.101
Butyric acid (mol %)[4]									
10.3[AB]	12.3[B]	9.05[A]	10.5	10.6	10.6	2.83	0.001	0.999	0.183

[1] n=6 rabbit/age and treatment, [2] germ counts expressed in $\log_{10}$ CFU/g caecal digesta, [3] expressed as μmol of reducing sugar / g dry matter of the caecal digesta / hour, [4] proportion within tSCFA, Different superscripts mean significant differences between [a,b,c] ages and [A,B] treatments. RSD = residual standard deviation, BW = body weight, DM = dry matter

Concerning fermentation traits (concentration and molar proportion of SCFA, pH-value of caecal content), our results of rabbits fed the inulin fed supplemented diet were unexpected and do not fit with the increased

total SCFA concentration (Volek et al., 2007), molar proportion of butyrate (Maertens et al., 2004) and lower pH found by these authors. In our experiment, medicated rabbits had lower molar proportion of acetic acid and higher of propionic and butyric acids than those fed inulin diet, or those fed control diet showing intermediate values. The lower molar proportion of butyric acid in animals fed inulin diet was in accordance with lower xylanase activity measured in their caecal content. This coincides with other findings according to which the end product of hemicellulose fermentation might be mainly butyric acid (Falcao-e-Cunha et al., 2004; Gidenne et al., 1998).

So the effect of inulin on rabbit caecal fermentation is not clear, and might be influenced by the composition of the diet, and the different large intestinal microbial population among rabbits (Carabano et al., 2006), not only in numbers but also in species composition. The ability to metabolise oligofructose and inulin is a strain-specific feature, so the effect of a prebiotic is influenced by the host's specific situation, as shown in case of *Lactobacillus* and *Bifidobacteria*. In addition, if a particular organism initiates metabolism of an oligosaccharide via extracellular hydrolysis, the products (mono- or disaccharides) that are released may then ''cross-feed'' other organisms (Huebner et al., 2007). The best known nutritional effect of inulin in humans (Niness, 1999) and in livestock (pig, poultry, calves, Verdonk et al., 2005) is its action to stimulate *Bifidobacteria* growth in the intestine. This effect is thought to promote health of the host: inhibiting the growth of harmful bacteria stimulation of the immune system, etc.

According to several investigations *Bifidobacteria* are not dominant in rabbit ceacum (Abecia et al., 2005; Monteils et al., 2008). In rabbits, there is a clear prevalence of the strictly anaerobic, non-sporulated gram-positive bacteria (Carabano et al., 2006, Monteils et al., 2008).

Other *in vivo* studies regarding the effect of the addition of inulin or oligofructose to the diet on the composition of the human colon microbiota reveal that *Bacteroides* are neither stimulated nor depressed by administration of these prebiotics (Roberfroid, 2005).

The relative lack of effect of inulin might also be accounted for by its partial hydrolysis in the upper intestine in rabbits, as observed previously by Maertens et al. (2004) due to the significant microbial activity in the intestine proximal to the caecum (Marounek et al., 1995), as occurs with other less fermentable sources of fibre (Carabano et al., 2001). However, whether the inulin had been partially hydrolysed before reaching the hindgut was not investigated in our experiment.

Medication resulted in the lowest morbidity as it could be expected, due to the inhibition of pathogenic bacteria (Commission on Antimicrobial Feed Additives, 1997), although no effect was detected on microbial counts. The increase in morbidity one week after weaning could be the result of the temporary increase in pH, *E. coli* and total aerobic bacteria number, which is a commonly observed accompanying feature of weaning and may predispose to digestive disorders (Lelkes and Chang, 1987). It has been shown in vitro that the antibacterial effect of SCFAs on *E. coli* is detectable up to pH 6.6, while it is completely absent above 6.8 (Prohaszka, 1986). This is because at lower pH, a higher proportion of SCFA is in non-dissociated form, which provides the antibacterial effect (Lewinson, 1978).

The increase in the relative weight and dry matter content of the caecal content with age is in accordance with the increasing importance to the large intestine after weaning, with increasing solid feed intake (Gidenne and Fortun-Lamothe, 2002). The enhanced fermentation activity in the caecum with age was shown by the increasing fibrolytic enzyme activities from 28 to 42 days of age. The fibrolytic potential of the caecal flora seems

to evolve weekly, however these enzymatic parameters are variable (Gidenne et al., 2002). Fibrolytic activity is high for pectins and hemicellulose (xylane) and lower for cellulose (Gidenne et al., 2002; Marounek et al., 1995) as it was observed in the present experiment as well. It was also supported by microbiological enumeration as higher counts of pectinolytic and hemicellulolytic stricktly anaerobic bacteria (mainly *Bacteroides*) were observed (Boulharouf et al., 1991). We found higher xylanase than pectinase activity, contrary to Volek et al. (2007), but comparing the composition of the experimental diets, our diets contained higher hemicellulose (NDF-ADF) (19 vs. 14%).

Total SCFA concentrations did not change with age, the SCFA profile was characteristic of rabbit, with the predominance of acetate, followed by butyrate and then by propionate (Padilha et al., 1995). The propionate to butyrate ratio decreased by age from 0.9 to 0.6, in accordance with the literature (Gidenne et al., 2002).

Microbial counts were not in agreement with the changes observed in fibrolytic activity and SCFA concentration. This may be due to the lack of information about SCFA production and to the high uncertainty of culturing techniques in studying microbial community, because it reveals only 20-40% of the real bacterial richness (Suau et al., 1999). Analysis of 16S rRNA genes demonstrated that the caecum of the rabbit harbours 80-90% of unknown bacterial species (Abecia et al., 2005; Monteils et al., 2008), which may have effect on fermentation processes.

4.3.3. DNA based qualitative analyses

With the help of the CE-SSCP technique some new qualitative information about rabbit caecal bacterial community has been determined.

On a fingerprint profile the relative abundance of bacterial community in case of one sample (medicated group, 42. day) is demonstrated by Figure 6.
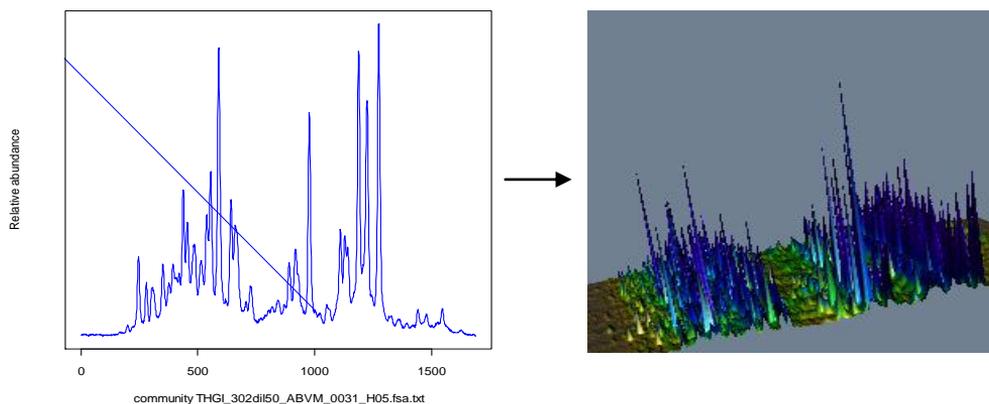

Figure 6: CE-SSCP fingerprint profile of caecal microbiota in a rabbit fed with medicated diet
Figure 7: Plotted microbiota of samples, all fingerprints

According to the principle of the fingerprint technique one bacterial species makes one peak in the profile. As about 40 peaks are visable, and it is supposed that there are more than 500 bacterial species in the rabbit caecum, due to coelution one peak represents several species. It means that this technic is not suitable for exact qualitative determinations, nevertheless profiles from different treatments or ages are comparable according to the diversity indexes. The differences or similarities of bacterial communities could be estimated. By plotted fingerprint profiles all samples (n=64) are demonstrable, with one bacterial community within one row.

Non-metric Multidimensional Scaling (nMDS) is a two dimensional display, where each fingerprint pattern is represented by a single plot. It respects the Euclidean distances between each pair of point. The effect of age could be demonstrated (Figure 8).

There is greater distance between black circles (28 d), than between red triangles (35 d). The smallest distances were calculated between green plus (42 d) signs.

The greater distance between two points means that is a bigger difference between bacterial communities of caecal bacteria. In other words, the points were layed on large area means that is big difference between bacterial communities. According to the 2D and the 3D visualisation, the tendency is that bacterial community more and more similar to each other in later ages.
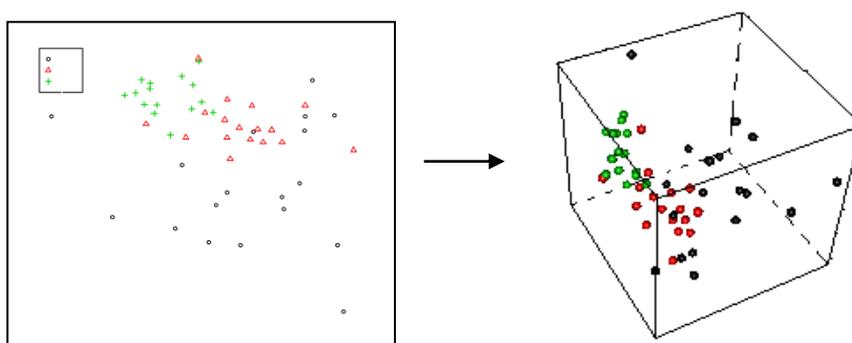
Figure 8: nMDS test - Effect of age, 2D and 3D visualisation
° 28 days, ^ 35 days, + 42 days; black: 28 days, red: 35 days, green: 42 days

According to the nMDS plots on Figure 9, diet had no effect on bacterial community as was shown by using the culturing technique as well.

Age effect (R=0.368, p<0.01) was supported also by Global ANOSIM, while diet had no significant impact (R=0.021, not significant) on the bacterial community.
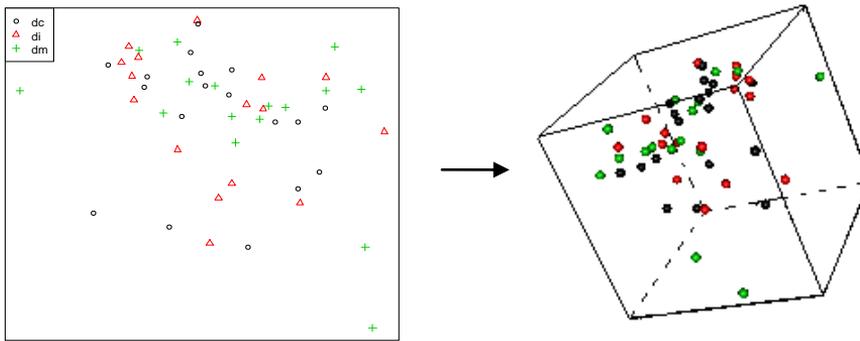
Figure 9: nMDS test - Effect of diet, 2D and 3D visualisation
dc: control -, di: inulin -, dm: medicated group; black: control, red: inulin, green: medicated

Pairwise ANOSIM was used to compare the structure of community in different ages. The results (R) were 0.166 and 0.486 when compared data of day 28 to 35 and 35 to 42 (<0.01), respectively. The highest R-value was calculated between 28 and 42 days of age (R=0.493, P<0.01).

Differences between fingerprint profiles of 28 and 35 and of 28 and 42 day old rabbits was compared by iterative test. More than a half of the profile was changed from 28 to 42 day of age (Figure 10).
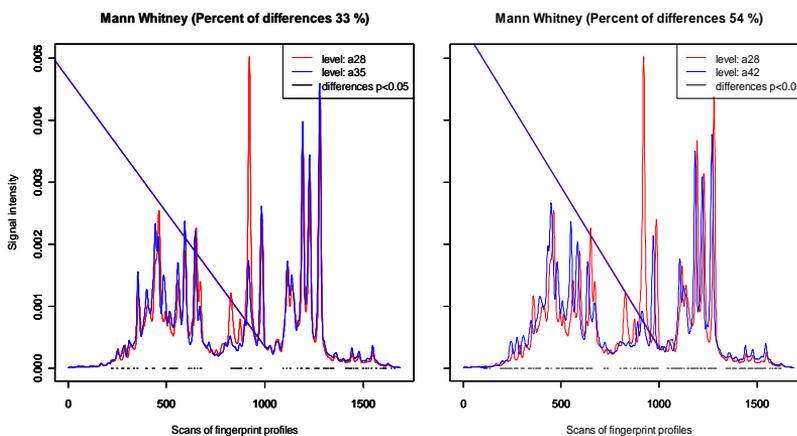
Figure 10: Iterative test – Differences between fingerprint profiles of 28 - 35 and 35 – 42 day old rabbits

Enzyme activity and volatile fatty acid concentration data were used to test the correlation between caecal bacterial community and the measured parameters. Only the concentration of propionic acid correlated significantly ($r = -0.407$, $p<0.01$) with the structure of community (Figure 11).
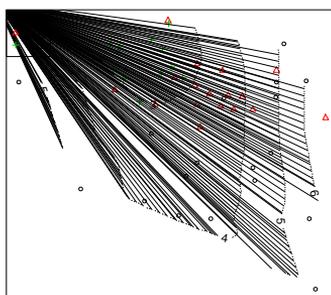
Figure 11: Relation between propionic acid and bacterial community
º 28 days, ^ 35 days, + 42 days

The Simpson index showed also age ($P<0.001$), but no diet effect (Table 17). The diversity indexes were increased from the beginning to the end of experimental period.

Table 17: Effect of diet and age on the bacterial diversity index in rabbits fed diets supplemented with antibiotics or inulin according to the capillary electrophoresis single strand conformation polymorphism (CE-SSCP) profiles

Experimental diets			Age (days)			P-value		
C	M	I	28	35	42	Diet	Age	Interaction
			Diversity index					
4.9 ±0.5	4.9 ±0.4	4.9 ±0.4	4.6 ±0.3[a]	4.9 ±0.4[b]	5.2 ±0.3[c]	0.445	0.000	0.323

Different superscripts mean significant differences between [a,b,c] ages.
C = control, M = medicated, I = inulin supplemented diet

4.3.4. DNA based quantitative analyses

According to the results of RT-PCR measurements, there were significant differences between ages in copy number of bacteria groups.

Copy number of total bacteria and *Bacteroides-Prevotella* decreased by 60.2% and 97.5%, respectively, between days 28 and 42 (Figure 12, 13).
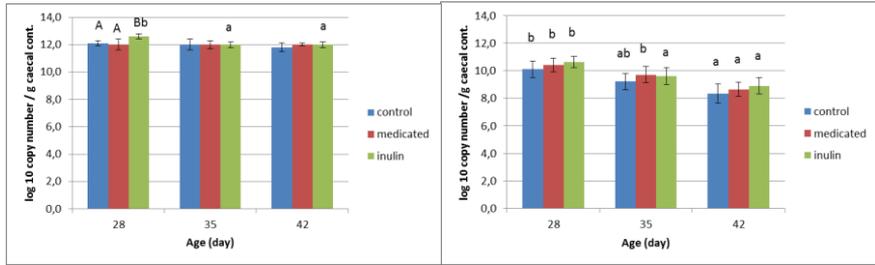

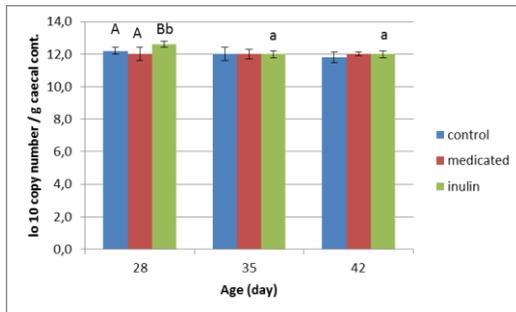**Figure 12: Effect of diet and age on copy number of total bacteria in caecal content of weaned rabbits (log 10 / g caecal content)**
Figure 13: Effect of diet and age on copy number of *Bacteroides-Prevotella* group in caecal content of weaned rabbits (log 10 / g)
Different superscripts mean significant differences between [a,b,c] ages and [A, B] diets..

Even the ratio of *Bacteroides-Prevotella* was less than 0.6% within total bacteria represented in the caecum on day 42 compared to data of day 28. The copy number of bacteria not belonging to *Bacteroidetes* group decreased from $\log_{10}$ 12.3 to 11.9 (Figure 14).

Figure 14: Effect of diet and age on copy number of Non *Bacteroides-Prevotella* group in caecal content of weaned rabbits (log 10 / g caecal content)
Different superscripts mean significant differences between [a,b,c] ages and [A, B] diets.

Inulin supplemented feed was available for the kits from day 21. Inulin could have andvantageous effect on microbiota during suckling (e.g. on *Lactobacilli and Bifidobacteria*) (Lynch et al., 2009; Passlak et al., 2012), which resulted in more total bacteria at weaning (at 28 days of age). Thereafter (on days 35 and 42) no significant difference attributable to inulin was detected. This resulted in significant diet x age interaction in copy number of total bacteria and not *Bacteroides-Prevotella* group (P=0.026 and 0.024).

Summarizing our data, 4% inulin supplementation of the feed between 21 and 42 days of age had no significant effect on caecal bacterial community, number of the measured bacteria, bacterial activity and the animal health status.

Maybe, inulin was partially digested in the upper GI tract and not in the hindgut (Maertens et al., 2004; Marounek et al., 1995), and it caused the invariable parameters of measured bacteria.

To exclude the effect of host – microbiota interaction, two *in vitro* experiments were carried out to clarify the effect of inulin on the microbial activity.

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

The results of the two experiments (effect of inulin supplementation and preincubation time on SCFA production and the composition of the microbiota) are summarised in Table 19 and 20.

The total SCFA production was 30% lower in samples supplemented with inulin compared to controls. Due to the inulin supplementation, the ratio of acetic acid (67.4%) and propionic acid (4.4%) was lower, while that of butyric acid (28.2%) was higher compare to control group (72.2%; 6.3%; 21.5%, respectively).

In a comparative *in vitro* work with oligosaccharides, inulin caused significant increase in acetate, propionate, but no significant change in butyrate concentrations (Rycroft, 2001). In an experiment connected by weaned rabbits fed with a diet in which starch was replaced by inulin, significantly higher concentration of SCFA was measured in the caecal content (Volek et al., 2005). The addition of inulin to rabbit feed significantly modified the molar proportions of SCFA, and characterized by a higher proportion of butyrate (Maertens et al., 2004).

Due to the inulin supplementation in Experiment 1, number of coliform bacteria was counted in higher (40%), while total anaerobic bacteria in lower amount (45%) in caecal content compared to control group. There was no significant difference between groups in the number of anaerobic bacteria.

According to the preincubation time, the concentration of tSCFA was increased with 175% from 0h to 12h. The coliform and the total aerobic bacteria number increased more than 150%. Total anaerobic bacteria number was decreased with 70% according to preincubation time.

In Experiment 1, there was a significant treatment x preincubation time interaction in the concentration of tSCFA. There was a significant increase in the control group between 6h and 12h preincubation time, while there was no significant difference in the same period in samples of inulin group. It confirms our previous results of *in vivo* experiment, in which there was no favorable effect of inulin on tSCFA production of caecal microbiota in adult rabbit.

The significant treatment x preincubation time interaction could be resulted by the high amount significant increase of total aerobic bacteria number (87.6 mmol/l) in control group between 6 to 12h preincubation period, contrary to the control treatment (Table 18).

Table 18: Effect of preincubation time and inulin supplementation on SCFA production and the composition of the microbiota in the *in vitro* experiment No. 1

Group		Preincubation time			SE	P-value		
C	I	0 h	6 h	12 h		Time	Group	Interaction
total SCFA (mmol/kg)								
85.57	59.93	50.10[a]	80.58[b]	87.57[b]	5.494	<0.001	<0.001	<0.001
Acetic acid (mol%)								
72.23	67.39	74.39[b]	67.78[a]	67.26[a]	1.048	<0.001	<0.001	0.123
Propionic acid (mol%)								
6.31	4.41	5.44	5.33	5.29	0.243	0.723	<0.001	0.148
Butyric acid (mol%)								
21.46	28.20	20.17[a]	26.88[b]	27.45[b]	1.199	<0.001	<0.001	0.061
Coliform ($\log_{10}$ CFU/g chyme)								
4.29	4.51	4.35[a]	4.31[a]	4.55[b]	0.049	0.037	0.009	0.055
Total aerobic bacteria ($\log_{10}$ CFU/g chyme)								
4.83	4.67	4.68[a]	4.68[a]	4.88[b]	0.046	0.030	0.003	<0.001
Total anaerobic bacteria ($\log_{10}$ CFU/g chyme)								
7.22	7.15	7.50	7.08	6.97	0.112	0.113	0.742	0.151

Different superscripts mean significant differences between [a,b,c] preincubation times.
Interaction between Group and Preincubation Time
C = control, I = inulin supplemented

In Experiment 2, there was no significant difference between inulin and control group in concentration of tSCFA. The proportion of acetic (67.3%) and propionic acid (5.3%) was smaller and butyric acid (27.5%) was bigger in I group compared to control (74.4%, 7.0% and 18.6%).

In this experiment, there were detected lower CFU number of coliform bacteria (56%) and total anaerobic bacteria (60%) in caecal content of rabbits fed with inulin supplementation feed, compare to control group (Table 19).

According to the preincubation time (at 0h and 12h), the tSCFA concentration was doubled. The proportion of acetic acid was decreased with 20%. Ratio of the propionic and butyric acid was increased from 4.5% and 16.5% to 7.1% and 28.9%, respectively.

In case of acetic acid and butyric acid proportion, there was significant treatment x preincubation time interaction detected. These could be resulted by the significant decrease the proportion of acetic acid and increase of butyric acid, in connection the preincubation time. Due to the inulin supplementation, the decrease of acetic acid ratio and the increase of butyric acid ratio were changed faster, than in control group.

In continuous anaerobic culture system using human faecal slurries *Bifidobacteria* preferred inulin, whereas *Bacteroides* could not grow on it. Wang and Gibson (1993) reported that after 12 h of incubation in the presence of 7g/L fructose, starch, inulin or oligofructose, both of the chicory fructo-oligosaccharides had a negative effect on growth of the *Bacteroides*.

A possible explanation for this might be related to the mechanism of inulin degradation. In case of *Bacteroides* it is presumed to be periplasmic or extracellular, causing loss of digestion products, while in contrast, in case of *Bifidobacterium* spp. enzymes are cell-associated or intracellular (Falony et al., 2009). As an important part of the rabbit caecal microbiota consists of *Bacteroides* especially before and around weaning (Combes, 2011, 2014; Monteils et al., 2008). This result may also provide an explanation of the lack of effect of inulin supplementation.

No consistent effect of inulin on caecal microbiota could be detected, but the concentration of acetic and propionic acid was decreased in our two *in vitro* experiments. The differences in the results between the two experiments can be probably attributable to the different age of the rabbits.

Table 19: Effect of preincubation time and inulin treatment on SCFA production and the composition of the microbiota in the *in vitro* experiment No. 2

Group		Preincubation time			SE	P-value		
C	I	0 h	6 h	12 h		Time	Group	Inter-action
Total SCFA(mmol/kg)								
68.76	65.35	36.60[a]	81.65[b]	82.92[b]	5.570	<0.001	0.432	0.440
Acetic acid (mol%)								
74.44	67.29	78.80[c]	69.69[b]	63.91[a]	1.873	<0.001	<0.001	<0.001
Propionic acid (mol%)								
6.99	5.25	4.51[a]	6.74[b]	7.12[b]	0.397	<0.001	<0.001	0.021
Butyric acid (mol%)								
18.57	27.46	16.50[a]	23.57[b]	28.98[c]	1.810	<0.001	<0.001	<0.001
Coliform ($\log_{10}$ CFU/g chyme)								
4.34	3.98	4.44[b]	4.18[ab]	3.86[a]	0.105	0.038	0.044	0.098
Total aerobic bacteria ($\log_{10}$ CFU/g chyme)								
5.44	5.37	5.36	5.45	5.41	0.079	0.922	0.678	0.398
Total anaerob bacteria ($\log_{10}$ CFU/g chyme)								
7.65	7.26	7.62[b]	7.59[ab]	7.15[a]	0.097	0.038	0.019	0.185

[a, b] = $p < 0.05$

Interaction between Group and Preincubation Time

5. CONCLUSIONS AND RECOMMENDATIONS

In rabbit production, the time around weaning is the most crucial period, when rabbits are highly sensitive to multifactorial digestive disorders. Weaning is usually performed between 28 and 35 days of age. Early weaning can be favourable by enabling lactating does and their kits to meet their different nutritional requirements. In contrast, the immature digestive system and low immune response, including the not-yet-balanced ecosystem of young animals, increase the risk of higher mortality and morbidity.

In our experiment age at weaning significantly influenced the growth of rabbits. In traditionally weaned (35 d) rabbits, milk consumption and the additional solid feed intake resulted in better growth. Significant changes were not affected by weaning in the counts of total aerobic bacteria, *Bacteroides* and coliform bacteria in caecal content, whereas it influenced SCFA production. Early weaning (21 d) did not produce considerable changes in the parameters of digestion 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.

Differing from pig and poultry production, where increasing standards of animal hygiene result in low antibiotic use, prevention programmes in rabbits are less productive. Facing this situation alternatives to antibiotics are intensively studied with a view to control enteropathies.

The supplementation of the does' diet with *Bacillus cereus* var. *toyoi* had a positive effect on production before weaning. This means that

supplementation of the does' diet with feed additives containing this microba strain was favourable regarding the growth of the kits when the same diet was fed 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 retardation in weight gain after weaning.

The main effect of probiotics is the improvement of the intestinal microbial balance of the host animals and the decrease of risk of colonization of pathogenic bacteria (competitive exclusion). Rabbits fed with the probiotic after weaning had significantly less coliform germ count in the caecum compared to other animals fed a diet without probiotic supplementation. This could be the reason for the better health status (significantly lower morbidity and mortality) and growth.

According to the results of our experiment, *Bacillus cereus* var. *toyoi* supplementation of the diet before and/or after weaning was beneficial related to health and production. But according to the Commission Implementing Regulation (EU) No 288/2013, the products containing *Bacillus cereus* strains were withdrawn from food and feed production of the European Union markets (EU Commission, 2013).

Among prebiotics the mannan oligosaccharides and the inulin is most frequently investigated in rabbits. Previously reported effect on performance of rabbits varied from little or no effect on weight gain, live weight and mortality, and/or increased caecal volatile fatty acid concentration.

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

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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 tSCFA 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.

The lack of consistency in these results is not surprising, as the gut microbiota is a very complex and yet not fully known ecosystem. Inulin as a probiotic substance should have developed its effect mainly by influencing the composition and the activity of the caecal microbiota (attributed to have bifidogenetic effect). The effect of inulin on rabbit caecal fermentation is not clear, and might be influenced by the composition of the diet, and the different large intestinal microbial population among rabbits, not only in numbers but also in species composition. *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 probiotic can not fully propose in rabbit meat production, more investigations into its mode of action and interaction with rabbit microbiota is needed.

Our experiments resulted in several new results and conclusions concerning development and growth of the digestive tract with age. The digestive tract of young animals is exposed to different changes around weaning. Anatomical development, introduction of microbial fermentation and caecotrophy, maturation of the digestive enzymes and the immune system are determined by ontogenesis but can be significantly influenced by weaning age and diet.

6.   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 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 qPCR.

7. SUMMARY

The major part of the breeding loss in commercial rabbit meat production is resulted by digestive disorders. 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, although there is an increasing human health and food safety concern over drug residues in meat products. In connection with the ban on using antibiotics as growth promoters in the EU, several studies have been carried out on different feed additives as alternatives to antibiotics.

The aims of these studies were to determine the effect of different weaning ages, to test the effects of probiotic *Bacillus cereus* var. *toyoi* and prebiotic inulin on the growth and digestion in the period around weaning, and to elaborate complex biomonitoring methods characterising the microbiota and caecal fermentation process in rabbit.

The effect of different weaning ages was examined in an experiment, in which three groups of kits (W21, W28 and W35) were formed according to the weaning age (day 21, 28 and 35).

On days 35 and 42, W35 rabbits had 10 to 14% and 10% higher BW, respectively, than those weaned at days 21 and 28. In the 4th week of life, early weaned animals had 75% higher feed intake than W28 and W35 rabbits. The relative weight of the whole GI tract was increased by 49% and 22% after weaning in W21 and W28 rabbits, respectively. Age related changes influenced the ratio of stomach, small intestine and caecum within the GI tract; however, no effect of different weaning age was demonstrated.

The concentration of total short chain fatty acids (tSCFA) was higher in W21 than in W28 and W35 throughout the experimental period. The

proportion of acetic and butyric acid within tSCFA increased, whereas that of propionic acid decreased, resulting in a C3: C4 ratio decreasing with age. Early weaning (W21) resulted in higher butyric acid and lower propionic acid proportions on day 28.

In conclusion, early weaning did not cause considerable changes in the digestive physiological parameters measured, but it resulted in 10% lower growth in rabbits.

Bacillus cereus var. *toyoi* is used as a probiotic feed supplement in livestock farming, where it has been reported to contribute to higher weight gain, improved feed conversion ratios, a reduction in the incidence of post-weaning diarrhea and lower mortality rates in several animal species.

The aim of the second experiment was to study the effect of *Bacillus cereus* var. *toyoi* (200 g/t in feed) on growth performance, caecal microflora and fermentation in rabbits.

Supplementation of the does' diet significantly improved the kit weight gain. The weight of control rabbits was significantly lower at 21 days weeks ages compared to treated animals. This could be presumably due to the better milk production of the does consumed the supplemented feed. The difference in body weight between the two groups was still marked on day 28.

The supplementation of the diet had a positive effect on production before weaning. The amount of anaerobic bacteria did not change, but the coliform count significantly decreased. This was presumably the main reason of the better production and health status. Morbidity and mortality were lower in animals fed the probiotic supplemented diet after weaning.

The aim of our *in vivo* inulin experiment was to study the effect of dietary supplementation of 4% inulin on growth performance and certain

digestive physiological parameters, especially the caecal ecosystem and the fermentation in weaned rabbits, in different ages.

Feed intake decreased in rabbits fed I diet compared to those fed M diet (by 11%), rabbits fed C diet showing an intermediate value. Growth rate from 28 to 35 days of age was not affected by diets, but decreased from 36 to 42 days in rabbits fed I diet compared to those fed C and M diets, with no effect on feed conversation ratio.

Inulin diet decreased caecal xylanase activity compared to C and M diets, reduced propionic and butyric acid and increased acetic acid concentration compared to M diet, whereas C diet showed intermediate values. The number of the strictly anaerobic bacteria decreased and cellulase and xylanase activity increased at 42 day of age compared to 28 and 35 days. Propionic acid concentration decreased with age from 28 to 42 day, but SCFA concentration and acetic and butyric acids proportions did not change.

In conclusion the inclusion of 4% of inulin in the diet of weanling rabbits showed no positive affect. To ascertain the interaction between inulin and rabbit caecal microbiota, two *in vitro* experiments were carried out to analyse the effect of incubation the caecal content with inulin, on the microbiota and SCFA production.

Ceacal samples taken from two adult rabbits (10 and 12 weeks old were preincubated with inulin, and after 0h, 6h and 12 hours of preincubation analysed for the composition of the microbiota and SCFA content.

In both experiments, total SCFA concentrations increased by 75 and 126%, respectively. The ratio (% within total SCFA) of acetic acid slightly decreased, while that of the propionic acid remained relatively constant in experiment 1 and increased in experiment 2. Due to the preincubation time, there was a definite and significant increase in the butyric acid ratio in both

experiments. No consistent effect of inulin on caecal microbiota could be detected, but the concentration of acetic and propionic acid was decreased in our two *in vitro* experiments.

8. ACKNOWLEDGEMENTS

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

10.1. Peer-reviewed papers published in foreign scientific journals

M. Kovács, Zs. Szendrő, G. Milisits, B. Bóta, E. Bíró Németh, I. Radnai, R. Pósa, <u>A. Bónai</u>, 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)

<u>A. Bónai</u>, 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, <u>A. Bónai</u>, 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)

10.2. Peer-reviewed paper published in Hungarian scientific journal

<u>Bónai A.</u>, 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)

10.3. Proceedings published in foreign language

M. Kovács, G. Milisits, Zs. Szendrő, H. Lukács, <u>A. Bónai</u>, 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.
Brescia: 701-704. (ISBN: 9788890281464)

<u>A. Bónai</u>, 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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<u>A. Bónai</u>, Zs. Szendrő, Zs. Matics, H. Fébel, R. Pósa, G. Tornyos, P. Horn, F. Kovács, M. Kovács: Effect of Bacillus cereus var. toyoi 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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<u>A. Bónai</u>, K. Horvatovich, V. Rajli, L. Kametler, M. Kovács: In vitro metabolism of inulin by rabbit microbiota. In: Hoy ST (szerk.) 16th International Symposium on Housing and Diseases of Rabbits, Furbearing

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10.4. Proceedings published in Hungarian language

Lukács H., Szendrő Zs., Bóta B., <u>Bónai A.</u>, Fébel H., Pósa R., Kovács M.: Eltérő korban történő elválasztás hatása a vakbélflóra összetételére és a fermentációra. (Effect of different weaning age on caecal microflora and fermentation in rabbits.) In: Szendrő Zs. (szerk.) 18. Nyúltenyésztési Tudományos Nap, Kaposvár, 2006. 05. 24. 139-145. (ISBN 963 86794 5 X)

<u>Bónai A.</u>, Szendrő Zs., Maertens L., Matics Zs., Fébel H., Kametler L., Tornyos G., Horn P., Kovács F., Kovács M.: Az inulin kiegészítés hatása a vakbélflóra összetételére és a fermentációra nyúlban. (Effect of inulin supplementation on caecal microflora and fermentation in rabbits.) In: Szendrő Zs. (szerk.) 20. Nyúltenyésztési Tudományos Nap. Kaposvár, 2008. 05. 21. 115-120. (ISBN: 9789639821057)

<u>Bónai A.</u>, Horvatovich K., Rajli V., Kametler L., Kovács M.: Inulin kiegészítés hatása nyulak vakbél ökoszisztémájára *in vitro* kísérletben vizsgálva. (Effect of inulin supplementation on caecal ecosystem in rabbits, *in vitro* experiment.) In: Szendrő Zs. (szerk.) 22. Nyúltenyésztési Tudományos Nap, Kaposvár, 2010. 05. 26. 97-101. (ISBN 978-963-9821-16-3)

11. OTHER PUBLICATIONS

11.1. Peer-review papers published in foreign scientific journals

M. Mwanza, L. Kametler, <u>A. Bónai</u>, V. Rajli, M. Kovács, M. F. Dutton: The cytotoxic effects of fumonisin B1 and ochratoxin A on human and pig lymphocytes using Methyl thiazol tetrazolium (MTT) assay. Mycotoxin Research, 2009. 25:(4) 233-238. (ISSN: 0178-7888)

11.2. Peer-reviewed paper published in Hungarian scientific journal

Kovács M., Sámik J., Fazekas B., Lelovics Zs., Pósa R., <u>Bónai A.</u>, Kovács F., Horn P.: Az ochratoxin-A kimutatása vesebeteg emberek véréből és vizeletéből. (Detection of ochratoxin-A from kidney disease human blood samples.) Acta Agraria Kaposváriensis, 2007. 11:(1) 1-9. (ISSN: 14181789)

11.3. Proceeding published in Hungarian language

<u>Bónai A.</u>, Dalle Zotte A., Vántus V., Morsy W. A., Kametler L., Matics Zs., Dal Bosco A., Szendrő Zs., Kovács M.: Spirulina és kakukkfű kiegészítés hatása a nyulak vakbélfermentációjára. (Effect of inulin and thyme supplementation on caecal fermentation in rabbits.) In: Matics Zs. (szerk.) 24. Nyúltenyésztési Tudományos Nap, Kaposvár, 2012. 05. 30. 111-115. (ISBN 978-963-9821-51-4)

12.   CURRICULUM VITAE

I was born in Pécs on 20 November 1982, and attended primary and secondary school in my native town. I obtained by General Certificate of Education in the 'Árpád Fejedelem' Secondary School in 2001.

Subsequently I gained admission to the Faculty of Animal Science of Kaposvár University. In 2004, I took an intermediate-level type C language exam in technical English (agriculture) and obtained an inseminator's diploma. In 2005, with my paper I took part in the 27th National Conference of Scientific Students' Associations. In the same year, I obtained advanced-level special qualifications as hatchery manager. In 2006, I acquired advanced-level game management qualifications and graduated from the University as a certified agricultural engineer. I took an entrance examination to the Doctoral School of Animal Breeding Science of Kaposvár University.

Between 2006 and 2009, I pursued my doctoral studies at the Faculty of Animal Science of Kaposvár University in the framework of a PhD programme in Animal Breeding Science. In 2008 I spent three months and in 2009 one month at the INRA-TANDEM Research Institute in France. I obtained my final pre-degree certificate in 2010.

Already as a first-year undergraduate, I had joined in the experimental work conducted at the Department of Physiology and Animal Hygiene. Since 2009, I have worked at that department and participated in the teaching of the subject Microbiology. In 2010–2011 I was a member of the Animal Breeding and Animal Hygiene Research Group of the Hungarian Academy of Sciences, and currently I am an Assistant Research Fellow in the 'Mycotoxins in the Food Chain' Research Group of the Hungarian Academy of Sciences in University of Kaposvár.