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The rabbit caecal microbiota: development, composition and its role in the prevention of digestive diseases – a review on recent literature in the light of molecular genetic methods

V. Bagóné Vántus, M. Kovács, A. Zsolnai
Kaposvár University, Faculty of Agricultural and Environmental Sciences
Physiology, Biochemistry and Animal Health Institute, H-7400 Kaposvár, Guba S. str. 40.

ABSTRACT

The caecum (and its microbiota) is essential to preserve the rabbit's digestive health. The intestinal microbiota is made up of hundreds of bacterium species, but only 25-40% can be cultured by the classical microbiological techniques. The aim of this paper is to summarize the development, composition and role of the caecal microbiota of rabbits; and the results of modern molecular biological methods.
(Keywords: rabbit, caecal microbiota, digestive health, molecular methods)

INTRODUCTION

The leading cause of mortality of kits is mainly due to diseases of the digestive apparatus (*Flatt et al*., 1974). Dysregulation of the intestinal mucosa homeostasis leads to a multitude of ailments mainly inflammatory bowel disease. The balance of the intestinal microbiota is essential to preserve the homeostasis. Facultative pathogens, primarily responsible for non-specific enteropathy, are activated by polyfactorial environmental elements (stress, nutrition).

The collection of microorganisms that live in peaceful coexistence with their hosts has been referred to as *microbiota*. Composition and roles of this bacterial community have been intensely studied in the past few years. Microbes can grow on the skin and in the genitourinary, gastrointestinal (GI), and respiratory tracts. By far the most colonized organ is the gastrointestinal tract, as it has a great surface area and rich in nutrients which can be used by microbes (*Sekirov et al*., 2010).

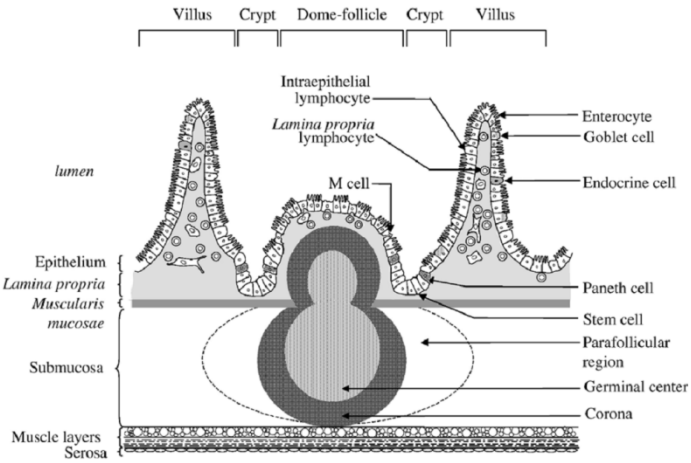
The rabbit's digestive tract is adapted to process large amounts of fibre rich feed, Microbial fermentation of the the food takes place in the caecum to ensure nutrient supply (*Harcourt-Brown*, 2004). Consequently caecal microbiota and fermentation processes play a key role in the digesting process, so it is an important issue to explore the gut microbiota, as well as factors affecting its composition and the development of rabbits. Besides pathogens the intestinal bacterial community imbalance (dysbiosis) is an important factor in the formation of digestive disorders and immune-mediated diseases (*Laparra and Sanz*, 2010). Gastrointestinal illnesses can cause death in 30-50% of the stock, and the animal's performance can also significantly be reduced (*Lelkes and Chang*, 1987).

The two main characteristics of the rabbit's caecal microbiota are 1) slow development (the first 3 days after birth is almost sterile in the appendix) and 2)

relatively simple composition (*Gidenne*, 1997). The caecum is colonised by microbiota forming bacteria, to protect against pathogens (binding sites on the coverage) and to facilitate gut development (mucosal histology, immune system) in early period of milk feeding. So the existence of normal microbiota in the gastrointestinal tract (GIT, gastrointestina) is an essential component of health in humans and economically important animal species. The lymphoid tissue is the place of interaction between the gut bacteria and the immune system; especially the gut-associated (GALT, gut associated lymphoid tissue) and mucosa-associated (MALT, mucosa associated lymphoid tissue) lymphoid tissue. The cell population of the mucosal digestive immune system contains diffuse cells between the epithelial cells and in the connective tissue (lymphocytes and plasma cells) or are organized in follicles and Peyer's patches

Figure 1

Schematic organisation of the gut-associated lymphoid tissue (GALT) (from *Fortun-Lamothe and Boullier*, 2007)



The intestinal microbiota is made up of hundreds of bacterium species, however only 25-40% can be cultured by the classical microbiological techniques (*Tannock et al*., 2000; *Suau et al*., 1999).

Based on the complexity of the gastrointestinal (GI) microbiota and the limited culturability of many of its members, it is clear that determining the function of the caecal bacterial community and its reaction to an applied treatment or environmental impact is a very challenging target.

The spread of microbial genomics methods has become increasingly evident as the living, unculturable, strictly anaerobic microbes may play an important role in the microbial metabolism and in the interactions between microbes and host. The majority of molecular techniques focussed on the 16S rRNA gene. These techniques often results in tenfold increase in microbe number compared to culturing procedures (*Carabano et al*., 2006). The spread of these techniques in recent years, changed the percepted image of the composition of the intestinal bacterial community. For instance Bacteroides were previously known as exclusively residential in the rabbit caecum (*Gouet and Fonty*,

1973) but after molecular analyses had been introduced their presence was reported to be only30% (*Abecia et al*., 2005, *Smith et al*., 2006). Due to these results and methods a new field of research has emerged designated as "Molecular Microbial Ecology'' having potential to provide a complete description and monitoring of the GI tract ecosystem.

The aim of this review is to expand our knowledge about the development, composition and role of the microbiota in the rabbit digestive system in the light of modern molecular genetic techniques. Promoting the feed efficiency of rabbits is as important, as reducing the incidence of digestive disorders.

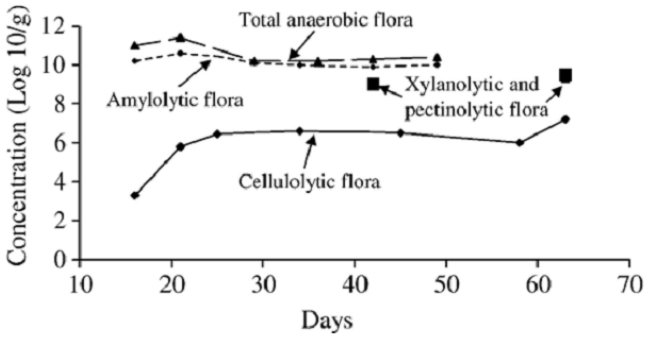
Rabbit caecal microbiota features, development and the possibility of control

Beside the contribution of the gastrointestinal tract microbial community to mammalian host health and performance, digestive microbiota is also involved in the supply of nutrients, especially in herbivorous species, the stimulation of the immune response and protection against pathogens (*Combes et al*., 2011).

In the rabbit's gut, the microbial colonization begins after birth, followed by gradual development of the intestinal microbiota. First the maternal gut flora colonise the infant gut (*Fortun-Lamothe and Boullier*, 2007). The foetus is normally sterile and is contaminated at birth with a heterogeneous collection of microorganisms from the birth canal and immediate environment (Berg, 1996). The key will be the first colonizing bacteria, which could influence steady-state gut flora composition in the adult individual (*Guarner and Malagelada*, 2003). When intake of solid food begins and a fibrous substrate enters the caecum, the colonisation of microbiota - involved in fibrolysis (hydrolysis of cellulose, xylanes, pectins, etc.) - starts (Figure 2). Huge fluctuation characterize the rabbit's bacterial community composition from the neonatal period (days7–28) until 3 weeks after weaning (day 49), the climax community -emerged by the process of ecological succession- is reaching steady state in young adult rabbits (days 70) (*Combes et al.*, 2011). In rabbits, our base knowledge of GI microbial community succession comes from culture-based study by Gouet & Fonty (1979).

Figure 2.

Establishment of the rabbit caecal microbiota between 2 and 10 weeks of age from (*Gidenne and Fortun-Lamothe*, 2002)



The bacterial composition of the individual can fluctuate under some circumstances. For instance: acute diarrhoeal illnesses or antibiotic treatment. Fluctuation can also be induced by dietary interventions, but individuals' flora composition pattern usually remain constant (*Simon and Gorbach*, 1984).

Control of the microbiota may give a chance to improve digestive efficiency, immune status and digestive health of rabbits. Improved digestive efficiency through optimization of the composition of the microbiota has direct impact; decreasing feed cost and increasing the use of fibrous raw materials which is necessary for fermentation processes (rabbit GIT adapted to process large amounts of fiber rich feed). In addition, improving digestive efficiency would reduce emissions to the environment. Finally, control of the microbiota could limit digestive disorders around weaning via its barrier effect and its role as an immune stimulator (*Combes et al*., 2013).

Roles of the intestinal microbiota

The composition and the activity of the caecal microbiota could have a strong influence on health, because of its role in nutrition, pathogenesis and immune function (*Gibson and Roberfroid*, 1995).

The role of the microbiota in the digestion and utilization of feed is manifested by hydrolysis of plant fibers and cell walls by bacterial enzymes, which is not possible by host animal digestive enzymes. The metabolic activities of the microbiota depend on the nature of incoming substrates and are organized in a digestive chain. The first step of this chain corresponds to the hydrolysis of complex polymers by a variety of hydrolases (polysaccharides, glycosidases, proteases, peptidases) provided by hydrolytic species to smaller compounds (monosaccharides, amino acids, etc.). These soluble compounds are used by hydrolytic and fermentative species as energy sources (*Combes et al*., 2013). Impact of the caecal ecosystem, on the overall digestive efficiency, derivable from the capacity of the microbiota provides 30% to 50% of maintenance energy requirements for an adult rabbit (*Gidenne*, 1992).

There is a host-microbiota symbiotic relationship, which defines the digestive ecosystem, where each partner benefits from the association. As for the rabbit: microorganisms colonize and grow rapidly under the favourable conditions of the gut, while the rabbit obtains the products of microbial fermentation from materials that could otherwise not be digested. In rabbits, this association is called a combined competition–cooperation model (*Mackie*, 2002). The balance of this ecosystem (eubiosis) is delicate and may be disturbed during digestive disorders: causing dysbiosis.

Caecotrophic animals – mammals, such as rabbits, lemurs, guinea pigs, chinchillas and hares – select the finer particles (<0.3 mm) leaving their caecum by antiperistaltic movements from the proximal colon and excrete them as soft faeces, that are reingested, as a way of recycling the protein from microbial origin. The most commercially important of these species is the rabbit (*Abecia et al.* 2005).

In rabbits, plant fibres are primarily digested in the caecum and the proximal colon, by the complex and very diverse microbial community inhabiting these two biotopes. E. coli and Clostridium spp. are the two potential pathogenic bacteria frequently present in diarrhoeic rabbits (*Peeters*, 1987). Bennegadi et al. (2003) found that Cellulolytic bacteria (*Ruminococcus albus, R. flavefaciens, Fibrobacter succinogenes* and *F. intestinalis*) represented <7% of total bacteria, with a predominance of *R. flavefaciens* and *R. albus*, respectively, for conventional and SPF (specific pathogen free) rabbits. Bacterial and archaeal rRNAs were twofold higher in conventional than in SPF rabbits,

fed standard diet ($p<0.001$). The caecal microflora of sick conventional rabbits (conventional and SPF) was markedly different from that of healthy conventional animals. All the microbial populations studied were affected except those of *R. flavefaciens* and the *Flexibacter-Cytophaga-Bacteroides* group. The decrease in these communities was probably linked to the reduction of the quality energy sources, because sick rabbits had reduced or stopped feeding.

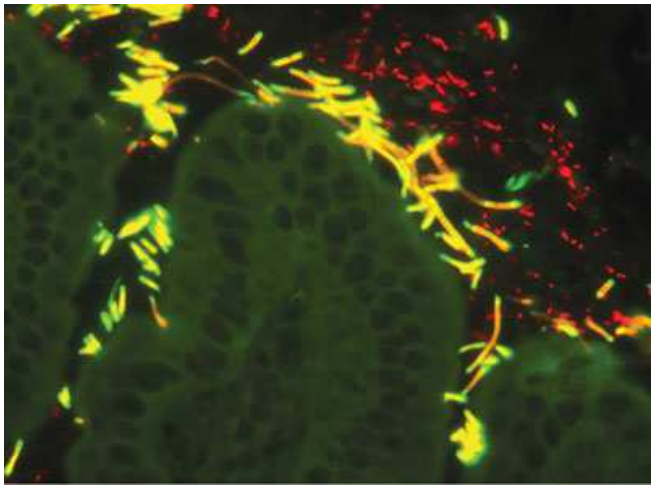
Besides their barrier function, microbiota is involved in immune organs and cell development, diversification of antibodies and mechanisms of oral tolerance.

The concept of barrier function is based on the fact that the microbiota permanently present in the digestive tract and inhibit the colonization of exogenous pathogenic bacteria (*Berg*, 1996). This takes place by (1) the adherence of commensal bacteria to the mucosa can prevent attachment and entry of pathogenic bacteria. For instance in rabbits, the segmented filamentous bacteria (SFB) (Fig. 3.) that colonize the ileum reduce the attachment of enteropathogenic E. coli (*Heczko et al*., 2000). (2) Competition among members of the microbiota for nutrients to maintain their ecological niche and habitat by consuming all resources. (3) By producing antimicrobial substances, to inhibit the growth of competing bacteria (*Guarner and Malagelada*, 2003).

Segmented filamentous bacteria (SFB), formally known as 'Candidatus Arthromitis', is the name given to certain uncultivable spore-forming bacteria of the phylum Firmicutes, within the order Clostridiales. SFB are present in the intestinal tracts of various species, including mammals (e.g. rabbit) and birds, but are not commonly present in humans. These bacteria are some of the very few commensal organisms that have been shown to directly contact intestinal epithelial cells (Figure 3): SFB (yellow) can directly contact the intestinal epithelium (green), unlike the rest of the commensal microbiota (red), which is located in the intestinal mucus.

Figure 3.

Segmented filamentous bacteria (SFB) Image courtesy of N.H.S. and P. Teggatz, Medical College of Wisconsin, Milwaukee, USA; (from *Bevins and Salzman*, 2011)



Investigations of molecular genetic techniques in rabbit intestinal microbiota

Classical bacterial culture methods, fluorescent in situ hybridization (FISH), molecular fingerprinting, qPCR, microarray techniques, high-throughput 16S rRNA gene sequence analysis and metagenomic approaches can reveal the population structure of the microbiota from heavily colonized intestinal mucosa (*Stecher and Hardt*, 2008).

Since only 24 to 40% of the microbial species of the microbiota can be cultured in vitro (Tannock et al., 2000), molecular microbiology techniques are now used to provide more sensitive and accurate parameters for biodiversity and stability (*Takahiro et al.*, 2003). The comparison between data acquired from molecular procedures and classical methods is problematic. By using molecular techniques, the determined microbe number is ten times greater than it is obtained by culturing procedure (*Carabano et al.*, 2006).

PCR (polymerase chain reaction) is a technique in molecular genetics that permits the analysis of any short sequence of DNA (or RNA) even in samples containing only minute quantities of DNA or RNA. PCR is used to reproduce (amplify) selected sections of DNA or RNA for analysis. Previously, amplification of DNA involved cloning the segments of interest into vectors for expression in bacteria, and took weeks. But now, with PCR performed in test tubes, it takes only a few hours. PCR is highly efficient so that extremely large numbers of copies can be made of the DNA. Main components used in test tubes are: Two "primers" (short single-stranded DNA sequences are synthesized to correspond to the beginning and ending of the DNA stretch to be copied). An enzyme called polymerase (several ones are available depending on the purpose of use) moves along the segment of DNA, reading its code and assembling a copy. A pile of DNA building blocks (nucleotides) needs to create new DNA strand.

DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases - adenine, guanine, cytosine, and thymine - in a strand of DNA. The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery. The first DNA sequences were obtained in the early 1970's (Maxam-Gilbert sequencing, Sanger sequencing) by using laborious methods based on two-dimensional chromatography. Following the development of fluorescence-based sequencing methods with automated analysis, DNA sequencing has become easier and faster.

New generation sequencing (high-throughput sequencing) technologies that parallelize the sequencing process, producing thousands or millions of sequences concurrently. Knowledge of DNA sequences has become indispensable for basic biological research, and in numerous applied fields such as diagnostic, biotechnology. The rapid speed of sequencing attained with modern DNA sequencing technology made achievable the sequencing of complete DNA sequences, or genomes of numerous types and species of life, including the microbial species.

After having information on DNA sequence PCR can be used as a sensitive technique to detect sequences, presented in very low concentrations, by species or group specific primers. More recently applied quantitative PCR method is the real-time PCR approach, which has been applied successfully to characterize GI samples from various species; this application looks promising because bacterial targets in very low concentration can be quantified, which is difficult using other (classical) approaches (*Zoetendal et al.*, 2004). The method may overcome the problem of quantifying very low concentrations of bacterial samples. While the quantification procedures may differ in

the quantitative power, the amount of 16S rRNA or ribosomes per cell itself is constantly changing (*Rigottier-Gois et al*., 2003a), and is influenced by factors such as bacterial species, the growth phase, the activity of the cell, and the differences in genome sizes and numbers of 16S rRNA gene copies per genome. (This perhaps renders extrapolation of the data to cell numbers inaccurate.)

Bennegadi et al. (2003) studied caecal community structure in conventional and specific pathogen-free (SPF) rabbits by performing dot-blot hybridization with **16S rRNA targeted oligonucleotide probes**. The variation of the caecal microbiota was analysed according to age, nutritional status and rabbit health (healthy or diarrhoeic). Caecal microbiota was stabilized at around 25–28 days. Bacteria and archaea represented 73% and 22%, of the total microbial communities at weaning (28 days), respectively. Cellulolytic bacteria represented <7% of total bacteria in conventional and SPF rabbits. This study shows some microbial interactions according to nutrition and health of the rabbit.

Abecia et al. (2005) found 46 new sequences from the 96 colonies picked as candidates for sequencing. The other clones were discounted because either only partial sequences were - cloned, the plasmid insert arising from primer dimers, or the insert failing to produce high quality sequencing (possibly due to more than one plasmid transforming the E. coli cell). These have been deposited in the EBI database with accession numbers AJ863512–AJ863557. Two of these sequences (clones 956 and 992) shared 99% identity, and another two (clones 948 and 986) shared 98% identity. All other isolates shared less than 97% identity. Based on a 97% identity threshold for species definition, Abecia et al. (2005) reports 44 novel species. Fragments of 16S rRNA genes were amplified from the extracted DNA by PCR using ‘‘universal’’ bacterial primers.

Monteils et al. (2008) constructed a bacteria library from the caecum of a conventionally held rabbit. **The complete gene 16S rRNA gene was sequenced.** The 228 clones obtained were distributed in 70 operational taxonomic units (OTUs). Units were distributed mainly (94%) in the Firmicutes phylum. Three sequences were related to Bacteroidetes. Nine clusters were defined in the phylogenic tree. A high diversity of caecal bacteria of the rabbit (comparable to equine large intestine or cow rumen) was shown indicating that herbivorous digestive ecosystems appear to have a strong diversity. Only one sequence had >97% similarity to cultured species; Variovorax sp. (identified in a soil ecosystem). All other sequences corresponded to uncultured bacteria. All sequences originated from digestive ecosystems (except Variovorax sp.) have high identity with sequences registered in the database. Among herbivores, the rabbit is a caecotrophic species, excreting two types of faeces, soft and hard, and consumes only the soft one. This behaviour improves the digestive efficiency of proteins and fibres through a valorisation of microbial protein of soft faeces. Therefore, the caecal microbiota plays an essential role in the rabbit digestive physiology, because of its size (40% of the whole tract content) and its highly active microbiota. The caecum evolved some specific characteristics (scale compared to other intestinal stage, histology, and fermentation) through adaption to caecotrphic lifestyle. Several of the sequences were similar to the sequences produced by *Abecia et al.* (2005) reinforcing the hypothesis that this caecum-specific species adapted to this particular biotope. Half of the sequences generated in *Monteils' et al.* (2008) library were distributed in the phylogenetic tree near the sequences characterized by *Abeica et al.* (2005) in rabbit caecum, suggesting that these can be considering as potential core species. The other half of the sequences were well separated (satellite species).

Michelland et al. (2010) aimed to study the response of the growing rabbit caecal ecosystem (bacterial community and caecal environmental parameters) after changing the conventional feed to a low-fibre diet (LFD). The bacterial community structure was characterized using **CE-SSCP** (Capillary Electrophoresis - Single Strand Conformation Polimorphism, a molecular fingerprinting technique) and total bacteria were quantified using **real-time PCR**. The reduction of fibre in the diet modified the CE-SSCP profiles ($P \leq 0.001$) but not the diversity index. The number of 16S rRNA gene copies of total bacteria decreased ($P \leq 0.01$) in LFD rabbits compared with controls. Significant correlations were found between the caecal bacterial community and its environment. These results suggest that the bacterial community in the growing rabbit caecum is able to adapt quickly after a change in the dietary fibre supply and able to reach a new steady-state equilibrium (modified structure of the bacterial community, and different amount of bacteria).

Combes et al. (2011) has analysed the development of the rabbit caecum microbiota and its metabolic activities from the neonatal (day 2) until the subadult period (day 70). The caecal microbiota was analysed investigating **16S rRNA gene** by **CE-SSCP** and **qPCR**. This study describes the microbial colonization process and provides a new insight into the dynamics of microbiota and in the establishment of stability. CE-SSCP showed, that the caecal microbiota developed from a simple and unstable community (found in neonatal stages) into a complex and climax community in young adult rabbit, which is also valid for the archaea colonization, which play essential role in the final stage of organic matter decay by reducing the carbon dioxide to methane.

Real-time PCR has been developed for monitoring the quantity of target sequence (part of bacterial DNA extracted from the sample) using probes or specific double-stranded DNA binding dyes e.g. SYBR Green. The bacterial quantification data using real-time PCR is commonly expressed as absolute quantities in units such as copies/g, colony-forming unit (CFU)/mL or Log CFU/g of samples. Quantitative PCR (qPCR) gives realtime monitoring, each cycle is detected via light (monochromatic or narrow wavelength range) exitation of a dsDNA binding fluorescent dye (SYBR Green): the intensity of the emitted light correlates with the amount of product generated by PCR, allowing quantitative detection (*Navidshad et al*., 2012).

CONCLUSION

The extremely diverse bacterial populations inhabiting the hosts GIT have an important role in many metabolic and immune processes and have a significant impact on the host's nutritional and health status. Metabolic activity, which expressed by the intestinal microbiota contributes to the digestion of food ingredients and energy storage it has also a role in micronutrient supply, and in the transformation of xenobiotics. Overall, the intestinal microbiota composition balance has a number of benefits for the host, while when the microbial metabolic balance is disrupted, it causes immune-mediated disease.

The caecal microbial community has been mainly studied using culture techniques, but such techniques reveal only 20–40% of the real bacterial richness. Few - before mentioned - studies used culture-independent analysis of 16S rRNA genes. They demonstrated that the rabbit's caecum harbors 80–96% of unknown bacterial species and contains no anaerobic fungi and a great proportion of archaea. The bacterial community contained a majority of Firmicutes (93%) and Bacteroidetes (4%). Molecular microbiology techniques are definitely useful, to provide sensitive and accurate identification of bacteria living in the digestive system of rabbits, the detection of

pathological lesions of gastrointestinal diseases and monitor treatment. The spread of molecular procedures in recent years, is "reshaping" the former image of the composition of intestinal microbiota.

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Corresponding author (*levelezési cím*):

Bagóné Vántus Viola
Kaposvár University, Faculty of Agricultural and Environmental Sciences
Physiology, Biochemistry and Animal Health Institute
Kaposvári Egyetem, Agrár-és Környezettudományi Kar
Élettani, Biokémiai és Állategészségügyi Intézet
7400 Kaposvár, Guba S. u. 40.
e-mail: violka36@gmail.com