EFFECT OF ADDING DIETARY CAPRYLIC ACID ON THE BACTERIAL POPULATION IN THE RABBIT CAECUM AND STOMACH

McEwan N.R.[1]*, Skřivanová E.[2], Worgan H.J.[1], Pinloche E.[1], Newbold C.J.[1], Marounek M.[2]

[1]Institute of Rural Sciences, Aberystwyth University, Aberystwyth, SY23 3AL, Wales
[2]Department of Animal Nutrition, Institute of Animal Science, Prague, Czech Republic
*Corresponding author: nrm@aber.ac.uk

ABSTRACT

The effect of dietary supplementation with caprylic acid on the bacterial population of the rabbit caecum and stomach was investigated using two different PCR-based methods: Denaturing Gradient Gel Electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphisms (TRFLP). Caprylic acid was added to the diet either in the form of 5 g/kg of the pure acid, or within Akomed R at 10 g/kg (with or without lipase at 10 g/kg). Rabbits fed *ad libitum* on their respective diets for 27 days before being euthanized and the digestive contents of the caecum and stomach removed for DNA analysis.

Neither analytical method suggested a change in the bacterial populations in the stomach dependent on the feeding group of the animal (control, caprylic acid, Akomed R or Akomed R and lipase supplementation). However, the use of DGGE suggested that the caecal samples could be split into two groups; those with no additional fatty acid supplementation of the diet, or where lipase was added along with the fatty acids; and the other where there was caprylic acid added either in its pure form, or in conjunction with other fatty acids in the form of Akomed R supplementation. Furthermore, there was an apparent increase in the detectable biodiversity following supplementation with either caprylic acid or Akomed R. No such segregation between dietary regimes was detected by TRFLP analysis. This is suggestive of there being a shift in relative numbers of specific organisms (DGGE data) without this effect being restricted specifically to one large-scale taxonomic group of organisms (TRFLP data). Thus dietary supplementation with caprylic acid, either in a pure form, or in conjunction with other medium-chain fatty acids, has the ability to target specific groups of microbes, whilst allowing a relatively large-scale bacterial diversity to persist.

Key words: Bacterial populations, Stomach, Caecum, Rabbit, Caprylic acid.

INTRODUCTION

The antimicrobial activity of medium-chain fatty acids (MCFA), containing 8 to 14 carbon atoms, and their monoglycerides has been studied extensively in recent years (reviewed by Thormar and Bergsson, 2001). However, the mode of action of MCFA in the animal digestive tract is not fully understood. Smith (1965) found the stomach and small intestinal contents of weaned rabbits were almost sterile. It was suggested that this was due to the rabbit milk fat containing antimicrobial compounds, identified as caprylic and capric acid (Canas-Rodriguez and Smith, 1966). In rabbits, they are synthesized *de novo* in the mammary gland, representing around 50% of the total fatty acids in milk (Jones and Parker, 1981). A protective role of rabbit milk was demonstrated in rabbits experimentally infected with enteropathogenic *E. coli* – EPEC - (Gallois *et al*., 2007). Caprylic acid had no effect on the growth rate of rabbits, but decreased mortality in the post-weaning period (Skřivanová and Marounek, 2002). Supplementation of the rabbit diet with caprylic acid and triacylglycerols of caprylic and capric acid decreased bacterial shedding in rabbits experimentally infected with EPEC O103 (Skřivan *et al*., 2005; Skřivanová *et al*., 2007).

The aim of the present study was to evaluate, if the supplementation of rabbit diet with MCFA affects bacterial diversity in the stomach and caecum of weaned rabbits.

MATERIALS AND METHODS

Animals and experimental design

Twenty weaned Hyplus rabbits of both sexes, 30 days of age, with initial weights of 771 ± 31 g were purchased from a commercial rabbitry (Dvory, Czech Republic). All nutritional experimentation was carried out at the Institute of Animal Science (Prague, Czech Republic).

Animals were randomly divided into four groups and housed in individual metabolic cages in an environmentally controlled stable. Rabbits in Group 1 were fed a commonly-used commercial mixed feed. Group 2 received the diet supplemented with caprylic acid (Sigma-Aldrich) at 5 g/kg. Rabbits in Group 3 were given basal diet supplemented with Akomed R (Karlshamns, Sweden) at 10 g/kg. Group 4 received the diet supplemented with Akomed R (10 g/kg) together with lipase (Texazym PES) at 10 g/kg. Caprylic acid and Akomed R supplementation was compensated for by rapeseed oil in the basal diet. All diets were antibiotic-free, supplemented with salinomycin, and pelleted. Rabbits were given diets *ad libitum* for 27 days, and then slaughtered.

Table 1: Ingredients and determined chemical composition of the basal rabbit diet

Ingredients (%)		Chemical composition (%)	
Alfalfa meal	30	Dry matter	91.1
Sunflower meal	13	Crude protein	17.7
Soybean meal	2	Fat	3.8
Wheat bran	26	Ash	8.0
Sugar beet pulp	4	Starch	15.9
Oats	6	NDF	40.1
Barley	14.5	ADF	18.0
Rapeseed oil	1.5	ADL	3.4
Vitamin supplement[1]	1		
Dicalcium phosphate	0.5		
Limestone	1		
Salt	0.5		

[1]Per kg of supplement: Vitamin A: 1 200 000 IU, vitamin D_3: 200 000 IU, vitamin E: 5 g, vitamin K_3: 0.2 g, vitamin B_1: 0.3 g, vitamin B_2: 0.7 g, vitamin B_6: 0.4 g, niacinamide: 5 g, Ca-pantothenate: 2 g, folic acid: 0.17 g, biotin: 20 mg, vitamin B_{12}: 2 mg, choline: 60 g, lysine: 25 g, DL-methionine: 100 g, salinomycin: 2.25 g.

Molecular Analyses

Immediately after slaughter samples of stomach and caecal contents were removed and stored at -70°C until analysis by molecular techniques. Digesta was broken up by bead beating. The sample was beaten for 30 seconds at 50 x 100 rpm (maximum speed) in a Minibeadbeater™ (Biospec products Inc.). Two tubes were beaten for each sample, pooled and stored at –20°C. Following bead beating, DNA was extracted from the cells using the QIAGEN QIAamp® DNA stool mini kits (Qiagen Ltd., UK). The manufacturer's methodology was adhered to other detailed below. Firstly, samples were incubated at 95°C for 5 minutes to lyse all bacteria, including the Gram-positive bacteria (manufacturer's guidelines), and to maximise DNA retrieval yields. Samples were centrifuged at 13,000 *g* in a Minispin (Eppendorf AG) for 2 minutes, as this was found to improve the residue deposition which facilitated extraction. After lysis, DNA damaging substances and PCR inhibitors were removed by absorption to InhibitEX (QIAGEN). The supernatant was extracted following centrifugation (13,000 *g* for 3 minutes). The DNA purification methodology also included the addition of proteinase K, which digested any proteins in the supernatant. Binding DNA to a QIAamp spin column facilitated the removal of impurities and retrieval of pure DNA.

PCR was performed using "DGGE" bacterial primers 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG–3' and 5'-ATT ACC GCG

GCT GCT GG-3' (Muyzer et al., 1993), using the following conditions: 1 cycle (94°C for 5 min, 55°C for 1 min, 72°C for 1 min); 35 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min); 1 cycle (94°C for 1 min, 55°C for 1 min, 72°C for 7 min), with a cocktail of 50 ng DNA, in a 50 µl reaction mix 1 µM of each primer, 0.8 mM of dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, and containing 2.5 U of *Taq* DNA polymerase in 10 mM Tris-HCl (pH 9.0). DGGE was performed using the BioRad Decode Universal Mutation Detection System, following the manufacturer's guidelines. PCR products were loaded onto 8% (w/v) TAE polyacrylamide gels containing a 35-55% denaturant gradient (100% denaturant; 7 M urea and 40% (v/v) deionised formamide). Electrophoresis was performed at constant voltage (130 V) and temperature (60°C) for 5 h. Gels were then stained for 30 min with silver stain. DGGE profiles within the same gel (i.e. either from the stomach or the caecum) were compared as described previously (Abecia *et al.*, 2007). Each band position present in the gel was binary coded for its presence or absence within a lane, and each lane was compared using a similarity matrix. Trees were constructed using the Hamming Distance values generated for each comparison (indicating the number of bands that differed between lanes) as an input for the NEIGHBOR program (PHYLIP version 3.5; Felsenstein, 1989).

TRFLP was performed using bacterial-specific primers, 27F (5'-AGA GTT TGA TCC TGG CTG AG-3') and 1389R (5'-AGG GGG GGT GTG TAG AAG-3'), to amplify the bacterial 16S *rRNA* gene. The 27F primer had a cyanine label (Liu *et al*., 1997) to allow TRFLP analysis. Amplification was performed with a BIORAD MyCycler™ thermal cycler program: an initial 4 min denaturation at 94°C followed by 25 cycles of 1 min at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C. A final cycle of 1 min at 94°C, 1 min at 55°C and elongation for 5 min at 72°C completed the PCR. The reaction cocktail used throughout was: 0.05 U/µl *Taq* DNA polymerase (Promega); 1x manufacturer's reaction buffer; 1.5 mM $MgCl_2$; 0.25 µM of each primer; and 0.2 mM of each of the dNTPs. All reactions were carried out in a volume of 50 µl. PCR products were purified to remove contaminating salts, unincorporated dNTPs or polymerase. The DNA concentration was then determined, to ensure DNA concentrations used in subsequent analysis were uniform. Digestion was performed using *HaeIII*, *HhaI* and *MspI* (Promega), all following the manufacturer's instructions. Restriction digest products were also purified, as above, to ensure uniform concentrations of DNA for terminal fragment analysis. 20 µl volumes were applied to a CEQ™ 8000 genetic analysis system (Beckman Coulter Inc). Analysis within the range 60-640 nucleotides was performed. Fragments peaks were analyzed and aligned using the AFLP (amplified fragment length polymorphism) facility in the software. Terminal restriction fragment (TRF) size calculations were estimated by comparison with an internal size standard using an algorithm available in the analysis software. An exclusion parameter of at least 5% of maximal peak height was imposed in order that samples could be easier to analyze against baseline noise levels. Analysis of TRF samples was performed by use of two different algorithms; a combination of Hamming distances and UPGMA, and Principal Component Analysis (PCA). UPGMA analysis was performed using Neighbor within the PHYLIP suite of programs.

RESULTS AND DISCUSSION

DGGE analysis of the bacterial population of the samples from the stomach (Figure 1A) did not reveal any population differences. However, there was a split in the samples from the caecal population (Figure 1B), into two general clusters: one with no additional fatty acid supplementation of the diet, or where lipase had been added; and the other where there was caprylic acid in its pure form, or in conjunction with other fatty acids in the form of Akomed R supplementation. It is interesting to note that the branch lengths in these dendrograms are proportional to differences between the samples. Thus, there is an increase in the detectable biodiversity following supplementation with either caprylic acid or Akomed R. It is important to remember that this is not an increase in the absolute diversity, but rather in the detectable diversity. This is presumably due to the survival of some of the species which are present at a minor level, at the expense of some of those which had been generally more abundant.

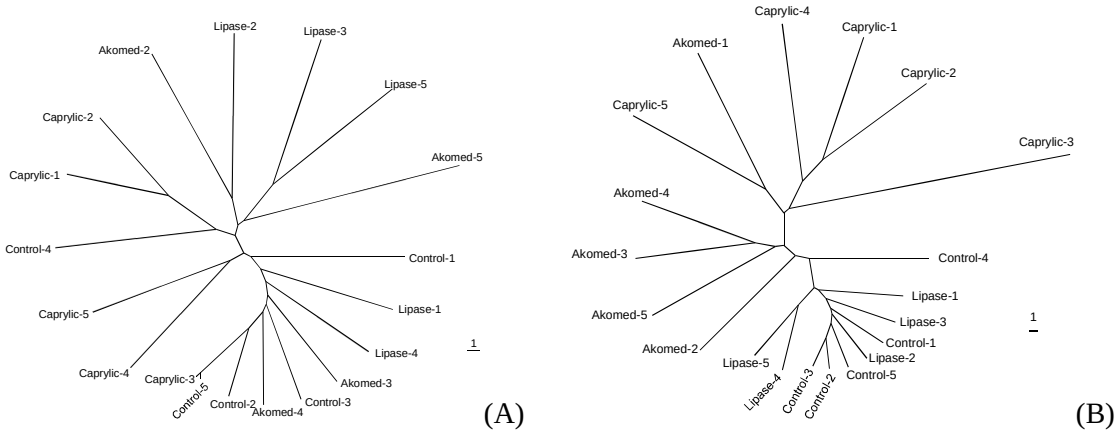

Figure 1: Bacterial diversity studies performed on digesta isolated from the stomach (A) and caecum (B) of rabbits using DGGE

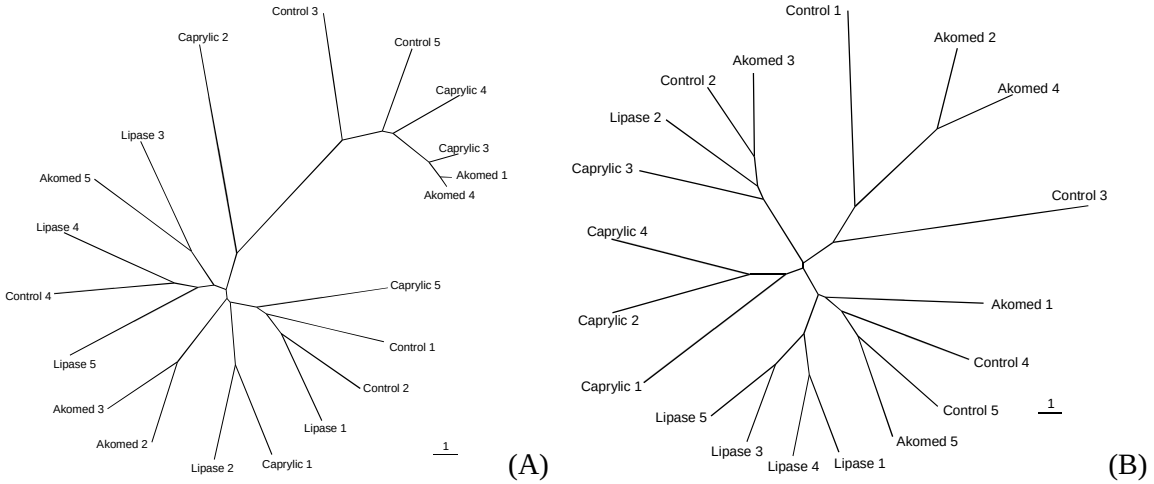

Figure 2: Bacterial diversity studies performed on digesta isolated from the stomach (A) and caecum (B) of rabbits using TRFLP

Using an alternative analysis, TRFLP, there was still no clustering pattern observed with samples from the stomach (Figure 2A). However, the caecal clustering effect with DGGE (Figure 1B) was not seen with TRFLP (Figure 2B). At first the DGGE and TRFLP results appear to conflict. However, it is important to consider the basis of the two techniques. DGGE relies on dissociating double stranded DNA as it moves through a gradient of increasing denaturant. This is what permits resolution between two fragments which differ by a single nucleotide. In relatively simple ecosystems, this implies that each band on the gel corresponds to a single DNA fragment. In a more complex ecosystem, such as the digestive tract, there are multiple sequences co-migrating within what appears to be a single band. Thus, different sequences of DNA with similar denaturing properties will appear to have similar mobilities on the denaturing gradient. This is already known, with multiple sequences being isolated from apparently single bands (e.g. Abecia *et al.*, 2007). Conversely, strands which co-migrate on the TRFLP columns share 5' terminal restriction fragment lengths and it is likely, although not implicit, that many of the co-migrating fragments are likely to have come from biologically related sources. This assumption is based on the premise that the probability of acquiring or losing a restriction cutting site in a particular piece of DNA is a relatively rare event. Thus, the two analytical methods are actually identifying two different things, with DGGE acting as a general indicator of changes in the population, and TRFLP acting as an indicator of changes between populations, by taking taxonomic relationship into consideration. Hence, there is a change in the population of bacteria based on the profiles determined by DGGE. However, it is interesting to note that the data generated by TRFLP imply that this is not something which can be attributed to the disappearance of a particular taxonomic group – based on the assumption that terminal fragment lengths are going to be conserved within a number, although not all, of closely related organisms.

CONCLUSIONS

Dietary supplementation with caprylic acid, either in a pure form, or in conjunction with other medium-chain fatty acids, has the ability to target specific groups of microbes, whilst allowing a relatively large-scale bacterial diversity to persist in the caecum.

REFERENCES

Abecia L., Fondevila M., Balcells J., McEwan N.R. 2007. The effect of lactating rabbit does on the development of the caecal microbial community in the pups they nurture. *J. Appl Microbiol., 103, 557-564.*

Canas-Rodriguez A., Smith W.H. 1966. The identification of the antimicrobial factors of the stomach of suckling rabbits. *Biochem J., 100, 79-82.*

Felsenstein J. 1989. PHYLIP - Phylogeny Inference Package (Version 3.2). *Cladistics, 5, 164-166.*

Gallois M., Gidenne T., Tasca C., Caubet C., Coudert C., Milon A., Boullier S. 2007. Maternal milk contains antimicrobial factors that protect young rabbits from enteropathogenic Escherichia coli infection. *Clin. Vaccine Immunol., 14, 585-592.*

Jones C.S., Parker D.S. 1981. The metabolism of glucose, acetate and palmitate in the lactating rabbit. Comp. Biochem. Physiol. B-Biochem. *Mol. Biol. 69, 837-842.*

Liu W.T., Marsh T.L., Cheng H., Forney L.J. 1997. Characterisation of microbial diversity by determining the terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl. Env. Microbiol., 63, 4516-4522.*

Muyzer G., de Waal E.C., Uitterlinden A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Env. Micr., 59, 695-700.*

Skřivan M., Skřivanová E., Marounek M. 2005. Effect of caprylic acid on health status of weaned rabbits experimentally infected with enteropathogenic Escherichia coli O103. *In: Proc. 3rd Rabbit Congress of the Americas, Maringà, Brazil.*

Skřivanová V., Marounek M. 2002. Effects of caprylic acid on performance and mortality of growing rabbits. *Acta Vet. Brno, 71, 435-439.*

Skřivanová E., Molatová M., Marounek M. 2007. Effects of caprylic acid and triacylglycerol of both caprylic and capric acid in rabbits experimentally infected with with enteropathogenic Escherichia coli O103. *Vet. Microbiol., doi: 10.1016/j.vetmic.2007.07.010.*

Smith H. W. 1965. The development of the flora of the alimentary tract in young animals. J. *Path. Bacteriol., 90, 495-513.*

Thormar H., Bergsson G. 2001. Antimicrobial effects of lipids. *Recent Developments in Antiviral Research, 1, 157-173.*