CHARACTERIZATION OF BACTERIAL COMMUNITIES IN CAEUM, HARD AND SOFT FECES OF RABBIT USING *16S RRNA* GENES CAPILLARY ELECTROPHORESIS SINGLE-STRAND CONFORMATION POLYMORPHISM (CE-SSCP)

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ABSTRACT

Dynamic and individual variability of caecal bacterial community were compared to those of hard and soft feces using capillary electrophoresis single strand conformation polymorphism (CE-SSCP). Soft and hard feces of 14 adult rabbits were weekly sampled during 5 weeks while caecal content was sampled on the 3rd (after surgery) and 5th week (after sacrifice). Bacterial communities were compared for their structure (peaks of CE-SSCP profiles analysis) and their diversity (estimation of Simpson diversity index on CE-SSCP profiles, D'). Bacterial community of caecal content, soft and hard feces presented individual variability ($P<0.05$ for diversity and structure). Without disturbance (1st to 3rd and 4th to 5th weeks), bacterial communities of soft and hard feces remained constant in time for diversity and structure. Sampling caecal content by surgical way greatly modified all bacterial communities ($P<0.05$ for diversity and structure) suggesting to find an alternative sample type to realize dynamic studies of bacterial caecal community within a same individual. The bacterial communities of the 3 types of sample were different ($P<0.05$), but presented a similar diversity (D'=3.96±0.48; NS). However, bacterial community of caecal content was closer to that of soft feces than that of hard feces (R of ANOSIM=0.13 and 0.24, respectively) and differed only on some peaks. Therefore, we can assume that soft feces could be used to realize dynamic studies of caecal bacterial community.

Key words: Bacteria, Caecum, Feces, CE-SSCP, Diversity, Structure.

INTRODUCTION

The caecum contains a dense and complex consortium of microbes mainly composed of bacteria which play a key role in the digestive physiology and health of the rabbit. Numerous studies were devoted to understand the relations between nutrition, digestive health and microbial activity. These works permitted to propose nutritional recommendations for fibre levels in relations with digestive health (Gidenne, 2003). Additionally, the characterization of caecal bacterial community could be helpful to understand origin of sanitary problems and to define new feeding or breeding strategies. Microbial communities were mainly studied using culture based methods (Fonty, 1974) and more recently using *16S rRNA* genes analysis (Bennegadi *et al.*, 2003; Abecia *et al.*, 2005; Abecia *et al.*, 2007; Cauquil *et al.*, 2007). New development of genetic fingerprint based on capillary electrophoresis and laser detection, like CE-SSCP, allow to quickly explore the whole bacterial community with high representativeness (Hori *et al.*, 2005). Therefore, this study aimed to explore dynamic and individual variability of bacterial community in the cæcum and to compare this flora with those of hard and soft feces using CE-SSCP to find an alternative sample type.

MATERIALS AND METHODS

Animals and experimental design

Samples were collected on 14 conventional rabbits (White New Zealand x Californian) aged of 12 weeks weighting 4.2 ± 0.5 kg at the beginning of the experiment. Rabbits were fed *ad-libitum* an experimental diet for 15 days before sampling until the end of experiment five weeks later. Diet did not contain any antibiotic or coccidiostatic and meet the nutritional needs of finishing-growing rabbit (18% crude protein ; 2.0% crude fat ; 14.2% crude fibre ; 19.4% starch ; 16.5% ADF). Samples were individually collected : i) cæcal content at 3rd (after surgery) and 5th weeks (after sacrifice of animal), ii) soft feces once a week from 1st to 5th weeks as previously described (Gidenne and Lapanouse, 2000) iii) hard feces once a week from 1st to 5th weeks.

PCR-CE-SSCP of *16S rRNA* genes

Total DNA was extracted using QIAamp® DNA Stool Mini kit (Qiagen). Genes coding for V3 region of *16SrRNA* were amplified by PCR with 30 cycles using primers W49 and 6-Fam-labelled W104. PCR and CE-SSCP conditions were previously described by Michelland *et al.* (2007). Briefly, CE-SSCP is a capillary electrophoretic method based on heterogeneity of single-strand ribotype secondary structure, providing different mobility through a non-denaturing gel. CE-SSCP produced chromatograms where bacterial communities were spread out on 1200 scan. Each peak of CE-SSCP profile usually corresponded to one bacterial species, but ribotypes co-migration events could occur and thus we assume that one peak correspond to ribotypes assembly (Zinger *et al.*, 2007).

Data handling and statistical analysis

Using Safum software in Matlab (Zemb *et al.*, 2007), chromatograms were first together aligned with a same reference internal standard and secondly normalized (area under bacterial community curve set to 1) to guarantee reliable sample comparisons. Statistical analyses were carried out in R software (R development Core Team, 2007).
Bacterial diversity was investigated by estimation of Simpson index (D') on each CE-SSCP profile (Haegeman *et al.,* 2008). ANOVA and Tukey's HSD post-hoc test were performed for D' using following fixed effects: rabbit (R1 to R14), week (weeks 1 to 5) and sample type (caecal content, hard feces and soft feces).
Bacterial structure: similarities between bacterial communities were investigated by pairwise comparisons of CE-SSCP profiles using Euclidean distances. Resulting proximities matrix were explored with non-Metric Multidimensional Scaling (nMDS) using 10000 random starts. Observed groups of CE-SSCP profiles were then statistically tested with Analysis of Similarity (ANOSIM). ANOSIM tests the significance of clusters existence with a p-value based on 10000 Monte Carlo permutations, and when significant, ANOSIM calculates the degree of difference between groups with an R-statistic from 0 (i.e. poorly differentiated) to 1 (i.e. well separated) (Ramette, 2007). When bacterial communities significantly differed, iterative ANOVA was performed for each of the 1200 points of CE-SSCP, using bacterial community membership (2 levels: community 1 or 2) as fixed effect.

RESULTS AND DISCUSSION

To our knowledge, bacterial communities of the rabbit gastrointestinal tract were never investigated using capillary electrophoresis fingerprints. Previous study using *16S rRNA* library sequencing suggested that caecal bacterial community is undoubtedly complex and harboured more than 99% of unknown species (Abecia *et al.*, 2005; Cauquil *et al.*, 2007). Therefore, the first step to study bacterial community of the cæcum is to explore its individual and time variability.

Variability of bacterial structure

Without disturbance, the bacterial communities of hard and soft feces did not differ (NS respectively for weeks 1 to 3 and weeks 4 to 5; Table 1). At the opposite, the sampling of caecal content using surgery induced significant changes in bacterial communities (R=0.29, P<0.001; Table 1).

**Table 1**: Similarity of bacterial communities among individual, sample type, time and surgery using ANOSIM

Sample origin groups	R-value	p-value
Rabbit	0.19	<0.001
Sample type	0.16	<0.001
Cæcal content vs. soft feces	0.13	<0.05
Cæcal content vs. hard feces	0.24	<0.001
Week	0.14	<0.001
1st, 2nd and 3rd weeks	-	NS
4th and 5th weeks	-	NS
Before vs. after surgical operation	0.29	<0.001

Therefore, the study of caecal bacteria community succession in response to disturbances (feeding or breeding factors) need an alternative sample type, easily accessible, non-disturbing for animal and microbes, and similar to caecal bacterial community. Both nMDS (Figure 1) and ANOSIM showed significant but barely separated CE-SSCP profiles according to sample type (R=0.16; P<0.05). However, ANOSIM showed that bacterial community of caecal content was closer to soft feces than to hard feces (R=0.13, 0.24 respectively, P<0.05).

Iterative ANOVA revealed that few peaks explained the structure difference between cæcal content and soft feces (Figure 2). Thus, in great majority, the same ribotypes assembly seemed to be observed in cæcal content and soft feces. These results suggested that it is possible to investigate dynamic of the cæcal bacterial structure by monitoring soft feces. ANOSIM showed individual variability (R=0.19; P<0.001) but pairwise comparisons of rabbits demonstrated that individual variability was mainly explained by few rabbits (data not shown).

Variability of bacterial diversity

Diversity of bacterial community of the cæcal content was meanly 3.8 ± 0.5 (Figure 3). Diversity varied among individual whatever sample type (P<0.001). Diversities of soft and hard feces did not change during the first 3 weeks (4.2 ± 0.4; NS), then decreased between weeks 3rd and 4th following surgery (-0.5 pt; P<0.05) and then did not changed between 4th and 5th weeks (3.7 ± 0.4; NS). Bacterial diversity of cæcal content and soft and hard feces was similar (3.75 ± 0.49, 3.94 ± 0.53; 4.08 ± 0.4 respectively; NS). There was no interaction between sample type and week effects.
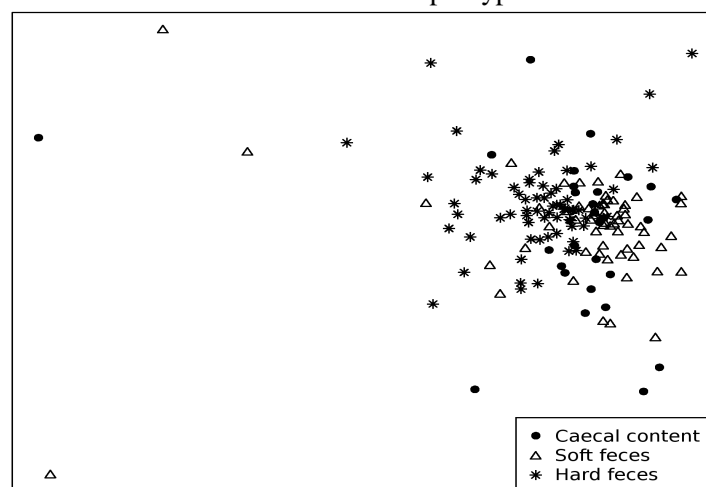
Figure 1: nMDS of CE-SSCP profiles according to the sample type
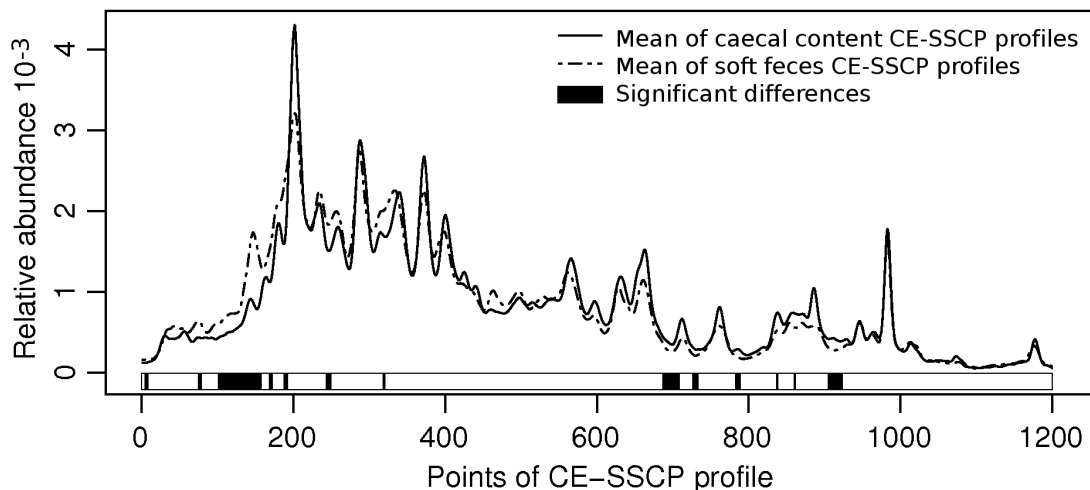
Figure 2: Structure differences on CE-SSCP profile between bacterial community of cæcal content and soft feces using iterative ANOVA. Significant differences at P<0.05 were indicated by black horizontal bars
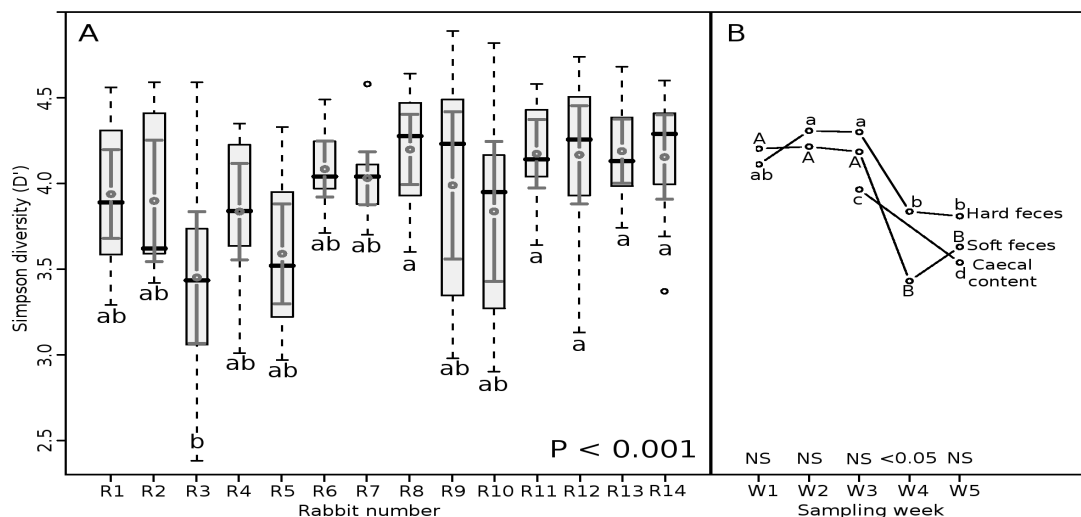
Figure 3: Boxplot and average of diversity for the 14 rabbits (A) and average of diversity by week and sample type (B)

CONCLUSIONS

Without disturbances, both diversity and structure of bacterial community of rabbit soft and hard feces were stable. Such a result allows studying caecal bacterial community changes in response to nutritional challenge comparing bacteria before and after disturbance. However, the access to caecal content is difficult and surgical method affects greatly both bacterial diversity and structure. Our data showed that soft feces, which are easily accessible, presented a similar structure community of those of caecal content. Thus, soft feces could be an interesting alternative sample type to study bacterial community of rabbit caecal ecosystem.

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