

Preliminary report on PCR detection of faecal microflora in *Prunella collaris*

V. KMEŤ, B. ŽATKOVIČ and P. PRISTAŠ
Institute of Animal Physiology, Slovak Academy of Sciences, Šoltésovej 4, 040 01 Košice, Slovakia

Abstract. We compared the diversity of chromosomal DNA isolated from Alpine Accentor (*Prunella collaris*) faeces collected from various locations in high mountains of Slovakia, Austria and Switzerland in two different years (1998 and 1999). The microflora was extracted, purified and analysed without cultivation. Bacterial 16S rDNA was amplified by PCR and the amplification products analysed by RFLP analysis. We consider this genetic approach a suitable method to characterise microbial community changes, because cultivation experiments only show the presence of a limited range of live bacterial species (*Enterococcus* spp. and *Enterobacter* spp.). Our results showed that the diversity of faecal microflora varied according to the year rather than the site of sample collection. In addition, using genus-specific primers, we showed the presence of *Bifidobacterium* spp. in faecal samples from the Alps.

Key words: Alpine Accentor, *Bifidobacteria*, faecal microflora, high mountains, PCR, *Prunella collaris*

Introduction

Considering the wide diversity of birds and the likely role of microorganisms in their nutrition, it is remarkable that, with only a few exceptions, very little attention has been given to the composition and activity of the avian gastrointestinal microbiota. The study of avian gut microbes can provide ornithologists not only with better understanding of forces influencing the relationship of this taxon with its environment, but also with a remainder that this taxon is itself an environment for other organisms.

For our study we selected the Alpine Accentor (*Prunella collaris*), a mountain bird living in high altitude environments, as our main avian research model for studying the interaction between commensal microflora and environment. Because it is a highly discrete bird whose sites of reproduction are far from human influence, there are few interactions between this species and humans. Perhaps only the development of ski resorts made it possible for accentors to begin over-wintering at high altitude with access to easily-obtainable food (Dyrz and Janiga 1997). We examined faecal samples from Alpine Accentors in high altitude chains of the Tatra Mountains, Slovakia, as well as in the Austrian and Swiss Alps.

Gastrointestinal microbial ecology has recently been experiencing something of a renaissance, in which classic culture-based microbial techniques are being supplemented, if not replaced, by molecular tools and techniques. Theoretically, molecular methods based on the 16S rRNA gene can now be used to identify all bacterial genera or species within gastrointestinal microbial communities, whereas cultivation approaches are biased due to the inability of some bacteria to grow on selective media, excluding them from further analysis (Simpson *et al.* 2000). This problem is of relevance to studies of gut microorganisms in samples of faeces from the Alpine Accentor, which are collected in high mountain conditions and during transportation to the laboratory usually only *Enterococcus* spp. or *Enterobacter* spp. remain alive. Therefore for monitoring the composition of faecal microflora we used a simplified method based on the 16S rRNA gene of bacteria (faecal bacterial community DNA analysis), in the sense that we subjected the resulting polymerase chain reaction (PCR) products to restriction endonuclease digestion and thus compared the bacterial community DNA fingerprints that arose.

Material and Methods

Faecal samples

Details of faecal samples collected in the Tatra Mountains, Slovakia, are shown in Table 1. Samples from the Austrian and Swiss Alps are shown in Table 2. Habitats were dominated by alpine meadows and rocky areas (Drgoňová and Janiga 1989, Nakamura and Ueuma 1996).

DNA isolation and agarose gel electrophoresis

Chromosomal DNA from faeces was isolated by sodium dodecylsulfate lysis and subsequent phenol-chloroform extraction and ethanol precipitation according to Wood *et al.* (1998) and the concentration of DNA was determined by UV spectrophotometry (A260nm). Purified DNA (c.100 ng) was used in the PCR reaction. PCR products were analysed by gel electrophoresis in 1.5% agarose gel containing ethidium bromide.

PCR

Approximately 100 ng of chromosomal DNA were amplified with a Techne Progene thermal cycler (Techne, Cambridge, U.K.) in a 50 µL reaction mix containing 0.04mM each dNTP, 20 pmol of each

primer, 1x reaction buffer and 1.0 unit PlatinumTaq DNA polymerase (Invitrogene). An initial denaturation step at 95°C for 5 min. was followed by 35 cycles of denaturation 94°C for 1 min., annealing 50°C (16S rRNA) or 62°C (*Bifidobacterium* spp.) for 1.5 min. and extension 72°C for 2 min. and a final incubation at 72°C for 8 min.

Primer composition

16S rRNA (Lane 1991) fD1: AGA GTT TGA TCC TGG CTC AG and rP2: ACG GCT ACC TTG TTA CGA CTT. Genus-specific primers for *Bifidobacterium* spp. Bif164 GGG TGG TAA TGC CGG ATG and Bif662 CCA CCG TTA CAC CGG GAA with product size 523 bp (Kok *et al.* 1996) After cycling, 10 µL of each reaction was analysed for the presence of PCR product on 1.5% agarose gel containing ethidium bromide at 7 V/cm.

RFLP

Product from 16S rRNA PCR was digested with HaeIII (BioLabs) restriction endonucleases. The products of digestion were analysed by agarose gel (3.0% w/v) electrophoresis (Amresco agarose 3:1, Solon, Ohio, U.S.A.) in TAE buffer.

Results

The potential of PCR based on 16S rRNA genes and subsequent restriction endonuclease digestion to discriminate between *Prunella collaris* faecal bacterial DNA was determined by comparing the banding patterns obtained. RFLP analysis showed a dominant bacterial group (unspecified) complex banding pattern of about seven bands in the range 100-1200 base pairs (Fig. 1). In the case of samples from the Tatra Mountains, the results showed two groups of chromosomal faecal DNA fingerprints (Fig. 2), with sample nos. 1 and 4 in one group and sample nos. 2, 3, 5 and 6 in the other. The division of the samples into two groups clearly corresponded to the respective year in which they were collected from the field rather than the location where they were found (Table 1). Local variability in faecal microflora of accentors from the Alps in the same year is shown in Fig. 3. Interestingly, the genera *Bifidobacterium* spp. and *Bacteroides* spp. were isolated from the alpine samples.

Discussion

From the point of view of the development of the avian gastrointestinal microflora, facultative anaerobic microorganisms, especially coliform bacteria and *Enterococcus* spp., are initially dominant, which create anaerobic conditions favouring the development of obligative anaerobes such as *Bacteroides-Prevotella* spp. as well as *Eubacteria*, *Clostridium* spp., *Peptostreptococcus* spp. and facultative anaerobic *Lactobacillus* spp. The normal bacterial flora in birds develops an effective defence mechanism against infection and illness. It is during times of illness, injury and excessive stress that the normal microflora, pH and digestive process can be affected. Examples

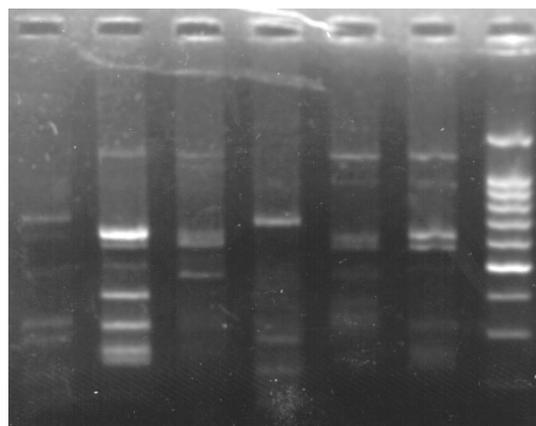


Fig. 1. Agarose gel electrophoresis of 16S rRNA RFLP PCR (*Hae*III)-generated fingerprints of faecal samples from *Prunella collaris* in the Tatra Mountains, Slovakia. The first six columns from left to right are the results from samples 1 to 6 respectively. See Table 1 in Material and Methods for details of sample collection localities and dates. The right-hand column is the standard.

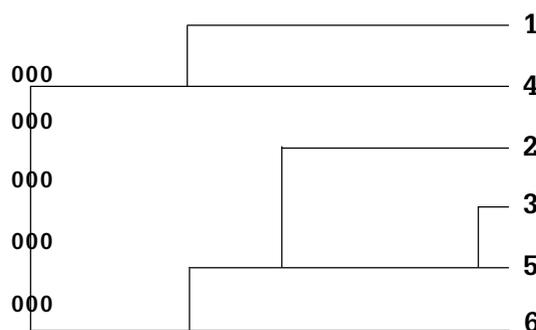


Fig. 2. UPGMA dendrogram showing DNA relatedness among six faecal samples (M1 to M6, corresponding to sample nos. 1 to 6 respectively in Table 1 and in the text) from Alpine Accentors in the Tatra Mountains, Slovakia, based on the Dice similarity coefficients. See Material and Methods for details of sample collection localities and dates.

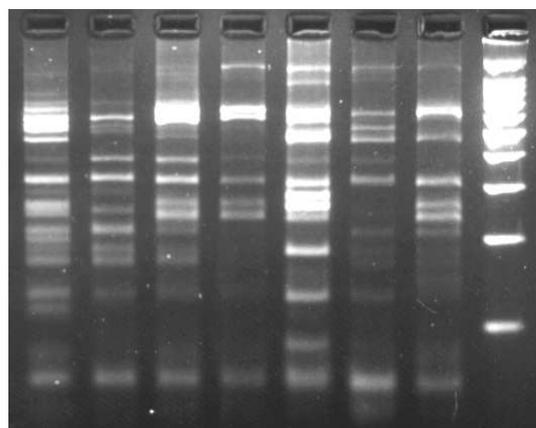


Fig. 3. Agarose gel electrophoresis of 16S rRNA RFLP PCR (*Hae*III)-generated fingerprints of faecal samples from *Prunella collaris* in the Austrian and Swiss Alps. The first seven columns from left to right are the results from samples 1 to 7 respectively. See Table 2 in Material and Methods for details of sample collection localities and dates. The right-hand column is the standard.

Sample no.	Bird	Locality	Height a.s.l.	Date
1	Juvenile	Malá Studená Valley lodge	2,015 m	11.10.1999
2	Unknown	Malá Studená Valley lodge	2,015 m	25.04.1998
3	Unknown	Skalnaté Tarn	1,751 m	08.04.1998
4	Unknown	Rysy peak	2,500 m	07.08.1999
5	Juvenile	Belianske Tatry ridge	2,000 m	01.07.1998
6	Unknown	Malá Studená Valley lodge	2,015 m	16.09.1998

Table 1. Faecal samples collected from Alpine Accentors in the Tatra Mountains, Slovakia

Sample no.	Bird	Locality	Height a.s.l.	Date
1 to 5	unknown	Seegrube-Hafelekar, Austrian Alps	2,330 m	21.05.1998
6	unknown	Hohgant, Swiss Alps	2,163 m	26.05.1998
7	unknown	Morgenberghorn, Swiss Alps	2,245 m	24.05.1998

Table 2. Faecal samples collected from Alpine Accentors in the Austrian and Swiss Alps.

of stress that can alter a bird's physiological microflora populations in the gut are: feed or formula changes, poor nutrition, hatching, weaning, inadequate or close housing, shipping, extreme temperatures, exposure to pathogenic microorganisms or viruses, a weakened immune system and overexertion (Alverdy and Stern 1998, Kleessen *et al.* 2000, McFarland 2000).

Microfloral composition is understandably influenced by host nutrition as well as the spatio-temporal circumstances in which faeces occur. According to the work of Janiga *et al.* (in prep.), date and year of collection are much more important factors for the composition of gut microflora than the location where host birds live. According to these authors, the bacterial microflora of Alpine Accentors in the Tatra Mountains, Slovakia, is specific not only among seasons but also among individual years, a conclusion supported by the results of the present study.

Of all the above-mentioned factors, probably seasonality has the greatest influence on the occurrence and diversity of gut microflora. Seasonality is an important aspect influencing the life-cycle of host species, particularly in alpine areas, where seasons are temporally shifted or shortened in comparison to localities at lower altitudes above sea level. Groups of microflora have been identified in Alpine Accentors, the seasonal occurrence of which is related to the life-cycle of the host (Janiga *et al.* in prep.). The degree of similarity among bacterial genera occurring in different samples is seasonally dependent: there is a higher similarity among samples from consecutive seasons, particularly autumn and winter, than among those from seasons separated by a quarter-year interval (Janiga *et al.* in prep.). The two groups of faecal bacterial chromosomal DNA fingerprints identified in the present study (Fig. 2) are consistent with this finding. In the first group were samples collected during summer and autumn of the same year, whereas samples in the second group were collected in a different year from spring to autumn. Similarity of samples compared between different years was not shown.

In our work we did not distinguish individual dominant groups of microorganisms. Instead we conducted an overall, unspecified analysis of the bacterial community in faeces of Alpine Accentors, which showed its diversity. We sought to test the suitability of genus-specific primers only for *Bifidobacterium* spp., which were present in all faeces collected from the Alps (data not shown). In the future we want to focus on the molecular detection of faecal microflora by the use of DGGE (denaturing gradient gel electrophoresis) or by analysis of bacterial DNA (Netherwood *et al.* 1999).

Acknowledgements

This investigation was supported by VEGA Grant Agency grant no. 2/4001/24 and by Science and Technology Assistance Agency grant no. APVT-20-026102. Fieldwork was conducted by Marián Janiga of the Institute of High Mountain Biology, Žilina University, Slovakia. We are grateful to Robin Rigg for translating and improving the manuscript.

References

- Alverdy, J. and Stern, E. 1998: Effect of immunonutrition on virulence strategies in bacteria. *Nutrition*, **14**: 580-594.
- Drgoňová, N. and Janiga, M. 1989: Nest structure of Alpine Accentors (*Prunella collaris*, Scop. 1769) in the Low Tatras. *Biologia* (Bratislava), **44**: 983-993.
- Dyrz, A. and Janiga, M. 1997: Alpine Accentor *Prunella collaris*. In *The EBCC atlas of European breeding birds: their distribution and abundance* (eds. Hagemeijer, E.J.M. and Blair, M.J.), p. 510. T & A D Poyser, London, U.K.
- Janiga, M., Sedlářová, A., Rigg, R. and Novotná M. in prep.: Patterns of prevalence among bacterial communities of Alpine Accentors (*Prunella collaris*) in the Tatra Mountains.
- Kleessen, B., Bezirtzoglou, E. and Matto, J. 2000: *Microbial ecology in health and disease* **12**(4): 53-63.
- Kok, R.G., De Wall, A., Schut, F., Welling, G.W., Weenk, G. and Hellingwerf, K.J. 1996: Specific detection and analysis of a probiotic *Bifidobacterium* strain in infant

- feces. *Appl. Environ. Microbiol.*, **62**: 3668–3672.
- Lane, D.J. 1991: 16/23S rRNA sequencing. In: *Nucleic acid techniques in bacterial systematics* (eds. Stackenbrandt, E. and Goodfellow, M.), p.115-175. John Wiley and Sons, Chichester, U.K.
- McFarland, L.V. 2000: *Microbial ecology in health and disease* **12**(4): 193-207.
- Nakamura, M. and Ueuma, Y. 1996: Comparative feeding ecology of the Alpine Accentor *Prunella collaris* on Mt. Hakusan and Mt. Norikura. *J. Yamashina Inst. Ornithol.*, **28**: 9-18.
- Netherwood, T., Gilbert, H.J., Parker, D.S. and O'Donnell, A.G. 1999: Probiotics shown to change bacterial community structure in the avian gastrointestinal tract. *Appl. Environ. Microbiol.*, **65**: 5134-5138.
- Simpson, J.M., McCracken, V.J., Gaskins, H.R. and Mackie, R.I. 2000: Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in faecal bacterial populations of weaning pigs after introduction of *Lactobacillus reuteri* strain MM53. *Appl. Environ. Microbiol.*, **66**: 4705-4714.
- Wood, J., Scott, K.P., Augustin, G., Newbold, C.J. and Flint, H.J. 1998: Estimation of the relative abundance of different Bacteroides and Prevotella ribotypes in gut samples by restriction enzyme profiling of PCR-amplified 16S rRNA gene sequences. *Appl. Environ. Microbiol.*, **64**: 3683-3689.

Received 3 March 2004; accepted 17 November 2004