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Streptococcus alactolyticus was the dominating culturable lactic acid bacterium species in canine jejunum and feces of four fistulated dogs.

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Abstract

Canine intestinal lactic acid bacterium (LAB) population in four fistulated dogs was cultured and enumerated using MRS agar. LAB levels ranging from $1.4 \times 10^6$ to $1.5 \times 10^7$ CFU ml$^{-1}$ were obtained in jejunal chyme. In the fecal samples $7.0 \times 10^7$ and $2.0 \times 10^8$ CFU g$^{-1}$ were detected. Thirty randomly selected isolates growing in the highest sample dilutions were identified to species level using numerical analysis of 16 and 23 S rDNA RFLP patterns (ribotyping) and 16S rDNA sequence analysis. According to these results, *Streptococcus alactolyticus* was the dominant culturable LAB species in both faeces and jejunal chyme. In addition, *Lactobacillus murinus* and *Lactobacillus reuteri* were detected.

Keywords: Culturable canine intestinal lactic acid bacteria, *Streptococcus alactolyticus*, jejunal chyme
Introduction

LAB are gram-positive, aerotolerant, catalase negative rods or cocci producing lactic acid as their main fermentation product. They form a heterogenous group of bacteria, the genera of *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus* and *Weissella* being the best known. Most LAB are non-pathogenic and they are associated with a wide variety of sources, such as plant material and various foods [1]. They also form a substantial part of the intestinal microbiota, and are believed to have a major effect on host’s well-being [2].

The knowledge of the canine intestinal LAB is scarce. Only few studies have previously addressed the canine intestinal microbiome [3-7]. Most of these studies date back to times when novel molecular techniques were not available and LAB were not identified to species level. Also the classification and nomenclature of LAB has been subjected to various changes during recent years.

In order to obtain knowledge of the culturable LAB species in canine intestinal microbiome, we enumerated and identified jejunal and fecal LAB associated with four permanently fistulated beagles. Culturing was done using anaerobic incubation and MRS agar and the predominating LAB species were identified to the species level using molecular methods.
Materials and methods

The dogs used in the study originated from the experimental animal colony of Helsinki University. They all had permanent jejunum nipple valve fistulas operated into the proximal jejunum, 60 cm distally from pylorus. The operations had been performed one to three years before this study took place according the method described by Wilsson-Rahmberg and Jonsson [8]. The fistulas did not cause any clinical discomfort or gastrointestinal symptoms to the dogs. The dogs had been used only for sampling of jejunal chyme and were not medicated. At the time of this study, the dogs were from three to six years of age. They were fed canned commercial balanced dog food, the main ingredients of which were cereal, meat, animal derivatives, oils and fats, vegetable protein extract and vegetable derivatives. The composition was as follows: raw protein 9 %, raw fat 6 %, raw fiber 0.4 %, calcium 0.3 % and phosphorus 0.25 %; moisture 80 %. The study had been approved by the Helsinki University ethics committee.

For the microbiological analyses, a sample of approximately 8 ml of jejunal chyme was collected from 4 permanently fistulated, healthy castrated male beagles 2 hours postprandial. Fecal samples were collected manually from rectum of two dogs. All samples were immediately submitted to the laboratory for microbiological analyses.

Samples were homogenized in 0.1% peptone water using a Stomacher blender. Serial 10-fold dilutions of the homogenized samples were made from $10^{-2}$ to $10^{-8}$ in 0.1% peptone water. LAB were enumerated on MRS agar (Oxoid, Basingstoke,
England) inoculated using the spread plate technique. All plates were incubated in an anaerobic CO\textsubscript{2} atmosphere (Anaerogen, Oxoid, 9-13\% CO\textsubscript{2} according to the manufacturer) at 30°C for 3 to 4 days. Five colonies from each sample were picked randomly from the plates showing growth of less than 100 colonies. Depending on the sample, these dilutions were \(10^6\times\) or \(10^7\times\) of the original sample. Isolates were cultured to purity using MRS agar/broth for species identification. Gram staining and catalase testing were performed before the molecular analysis.

Two ml of cultures grown overnight at 30°C in MRS broth were used for DNA isolation. DNA was isolated by guanidium thiocyanate method by Pitcher and others [9] as modified by Björkroth and Korkeala [10] by the combined lysozyme and mutanolysin (Sigma) treatment. \textit{HindIII} and \textit{EcoRI} enzymes were used for restriction endonuclease treatment of 4\(\mu\)g of DNA as specified by the manufacturer (New England Biolabs), and Restriction Endonuclease Analysis (REA) was performed as described previously [10]. Southern blotting was done using a vacuum device (Vacugene, Pharmacia), and the rDNA probe for ribotyping [11] was labelled by reverse transcription (AMV-RT, Promega and Dig Labelling Kit, Roche Molecular Biochemicals) as previously described [12]. Membranes were hybridized at +58 °C overnight, and the detection of the digoxigenin label was performed as recommended by the manufacturer.

For pattern analysis, the membranes were scanned with a Hewlett-Packard (Boise, Idaho, USA) Scan-Jet 4c/T scanner. The \textit{EcoRI} and \textit{HindIII} ribopatterns were compared with the corresponding patterns in the previously established LAB database at the Department of Food and Environmental Hygiene. Ribopatterns were analyzed
using the BioNumerics 3.0 software package (Applied Maths, Sint-Martens-Latem, Belgium). The similarity between all pairs was expressed by Dice coefficient correlation, and UPGMA clustering was used for the construction of the dendrogram. Based on the use of internal controls position tolerance of 1.5% was allowed for the bands. For the dendrogram combining the information from EcoRI and HindIII ribopatterns, equal weight was given to both banding pattern types.

Chromosomal DNA for use in PCR was isolated as for ribotyping. The nearly complete (at least over 1400 bases sequenced) 16S rRNA gene was amplified by PCR with a universal primer pair, 5’-CTGGCTCAGGAYGAACGCTG-3’ as the forward primer, corresponding to positions 19-38 in Escherichia coli 16S numbering, and 5’-AAGGAGGTGATCCAGCCGCA-3’ as the reverse primer, complementary to positions 1541-1522. Sequencing of the purified (QIAquick PCR Purification Kit, Qiagen) PCR product was performed by Sanger’s dideoxynucleotide chain termination method as two long and two shorter reactions. Samples were run in a Global IR$^{32}$ using LiCor sequencing device with e-Seq 1.1 software (LiCor) according to the manufacturer’s recommendation. Overlapping complementary sequences were joined by the Align IR 1.2 program (LiCor). Nucleotide sequence data were analyzed with version 32.0 of the BioNumerics software package (Applied Maths).

Phylogenetic analysis of the 16S rDNA sequence of strains was performed by using the Bionumerics 3.0 software package (Applied Maths). Calculation of the level of similarity and construction of a phylogenetic tree was based on the neighbour-joining method. Bootstrap probability values were calculated to branching points resampling 1000 trees.
Results

LAB levels ranging from $1.4 \times 10^6$ to $1.5 \times 10^7$ CFU ml$^{-1}$ were obtained in the jejunal chyme. In the two fecal samples, $7.0 \times 10^7$ and $2.0 \times 10^8$ CFU g$^{-1}$ were detected. All isolates were gram positive and catalase negative. Twenty of them possessed coccal morphology while 10 were rod shaped.

Three LAB species, *S. alactolyticus*, *L. murinus* and *L. reuteri* were detected by the means of the RFLP database and 16 S rDNA sequencing. Fig. 1 a and b show the dendrograms generated by *Eco*RI and *Hind*III restriction enzymes, respectively. Fig. 1c was made by combining the information from both restriction enzyme analyses together. All types of analyses resulted in species-specific clusters showing pattern similarity values ranging from 46.2 to 100%. In the distance matrix tree based on the 16S sequences (Fig. 2), strains were located in 3 branches corresponding well to the species-specific clusters obtained by ribotyping.

Table 1 shows the LAB species distribution within the 30 randomly selected isolates identified to the species level. Within a species, identical ribopatterns were obtained from the isolates by both enzymes used. Fig 1. shows the representative patterns of all different types obtained. *S. alactolyticus* was found to be the dominant LAB species isolated from both faeces and jejunal chyme. *L. murinus* was associated with 3 of the dogs while 2 dogs were found to carry *L. reuteri* (Table 1).
Discussion

S. alactolyticus was found to be the dominating culturable LAB species in the jejunal and faecal samples associated with the dogs in the present study. It was found in all the dogs and in every sample. In addition to S. alactolyticus, strains belonging to species L. reuteri and L. murinus were detected to a lesser extent (Table 1).

To our knowledge, this is the first report on the composition of the most prevalent culturable LAB species in the canine jejunal chyme and faeces. S. alactolyticus was described by Farrow and others [13], they isolated it from the intestines of pigs and the faeces of chicken. This organism has also been documented to reside in the pigeon intestines, although only as a minor part of the microbiota [14]. Ureolytic Streptococcus intestinalis was reported to be the predominant member of the pig colonic microbiota [15]. Later work by Vandamme and co-workers [16] revealed that S. intestinalis is a junior subjective synonym of S. alactolyticus and therefore pigeons must also be considered as a host of S. alactolyticus.

In a recent study [7], the faecal microbiota of four Labrador retrievers was examined, and S. bovis and L. murinus were found to be the most prevalent culturable LAB species. In this study, there was variation in the occurrence of LAB species between the different samples. This was not clearly evident in our work. However, it has been documented that the canine intestinal microbiota may change in time [6], so the finding could reflect natural variation. The composition of intestinal bacterial flora is known to be host species specific and dependent on dietary and environmental factors [17]. This may also explain the differences in LAB strains between the present
study and the work published by Greetham and co-workers [7]. In addition, their
study dealt only with the faecal microbiota whereas we identified the most prevalent
culturable small intestinal LAB, too. However, the dogs we studied live in a colony of
experimental animals. They have very few contacts with dogs outside the colony and
their lives do not fully resemble the life of a domestic pet. On the other hand, the
possibilities to examine the small intestinal microbiota in healthy, non-medicated pet
dogs are practically nonexistent.

LAB are reported to have several beneficial effects on host’s well being. They
may suppress the growth of intestinal pathogens by the means of competitive
exclusion [18, 19], and they have been documented to enhance the immune functions
in humans and mice [20, 21]. It is noteworthy that with the exception of *L. reuteri*,
none of the LAB strains detected in this study are used in commercial probiotic
products.

Human gut microbiome has already been studied using various culture-
independent methods whereas in association with canine intestinal microbiome these
studies are only on their way. Therefore, our results form a basis for the future either
culture-dependent or independent studies dealing with canine intestinal microbiota.
We conclude that knowledge of the dominant culturable LAB in the dog is necessary
for further studies on the canine intestinal microbial ecology.
Acknowledgements

We would like to thank Ms. Henna Niinivirta for the excellent technical assistance. Financial support from the Academy of Finland (project 100479) for the identification of the LAB strains is gratefully acknowledged.
References:


description of *Streptococcus alactolyticus* sp. nov. and *Streptococcus saccharolyticus* sp. nov. Syst. Appl. Microbiol. 5, 467-482.


Table 1. Species division (number of isolates) within the LAB 30 isolates cultured pure from jejunal chyme or feces of 4 castrated male dogs with permanent jejunum nipple valve fistulas. Species were identified by the means of a RFLP database and 16S rDNA sequencing.

<table>
<thead>
<tr>
<th>LAB species</th>
<th>Dog 1</th>
<th>Dog 2</th>
<th>Dog 3</th>
<th>Dog 4</th>
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<tbody>
<tr>
<td></td>
<td>jejunal</td>
<td>feces</td>
<td>jejunal</td>
<td>feces</td>
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<tr>
<td><em>Streptococcus</em></td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>3</td>
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<tr>
<td><em>alactolyticus</em></td>
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<tr>
<td><em>Lactobacillus</em></td>
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<tr>
<td><em>reuteri</em></td>
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<tr>
<td><em>Lactobacillus</em></td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>murinus</em></td>
<td></td>
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</table>
Fig. 1. (a), (b) and (c) present numerical analysis of 16 and 23S RFLP patterns (ribotypes) generated by EcoRI, HindIII and an analysis combining the information of both restriction enzymes, respectively. Clusters show representative patterns of all different types obtained. Numerical analyses of the patterns are presented as dendrograms, left side of the EcoRI and HindIII banding patterns possesses high molecular weights, < 23 kbp, and right side >1000 bp. Scales show percentual similarities of the patterns.

Fig. 2. Phylogenetic tree based on similarity values of almost entire 16S rDNA sequences (at least 1400 bp). Bootstrap probability values from 1000 trees resampled are given at the branch points. C. jejuni was used to root the tree. Scale shows 5% difference.
Fig. 1, Rinkinen et al.
Fig. 2, Rinkinen et al.