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

Minna L. Rinkinen[*], Joanna M.K. Koort[†], Arthur C. Ouwehand[‡], Elias Westermarck[*], K. Johanna Björkroth[†]

[*]Department of Clinical Veterinary Sciences, Faculty of Veterinary Medicine, P.O. Box 57, FIN–00014 University of Helsinki, Finland

[†]Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, P.O. Box 57, FIN–00014 University of Helsinki, Finland

[‡]Department of Biochemistry and Food Chemistry, University of Turku, FIN-20014 Turku, Finland

Corresponding author:

Minna Rinkinen, Department of Clinical Veterinary Sciences, Faculty of Veterinary Medicine, P.O. Box 57, FIN-00014 University of Helsinki, Finland

Tel: +358-50-549 78 38

Fax: +358-9-191 49 670

E-mail: Minna.Rinkinen@helsinki.fi

Abstract

Canine intestinal lactic acid bacterium (LAB) population in four fistulated dogs was cultured and enumerated using MRS agar. LAB levels ranging from 1.4×10^{6} to 1.5×10^{7} CFU ml^{-1} were obtained in jejunal chyme. In the fecal samples 7.0×10^{7} and 2.0×10^{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_2 atmosphere (Anaerogen, Oxoid, 9-13% CO_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 × or 10^7 × 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. *Hin*dIII and *Eco*RI enzymes were used for restriction endonuclease treatment of 4 μg 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 ºC 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 *Eco*RI and *Hin*dIII 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 *Eco*RI and *Hin*dIII 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.

142 Results

143

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

148

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

157

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

164

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.

212 Acknowledgements

213

214 We would like to thank Ms. Henna Niinivirta for the excellent technical

215 assistance. Financial support from the Academy of Finland (project 100479) for the

216 identification of the LAB strains is gratefully acknowledged.

217

References:

[1]. Axelsson, L. (1998) Lactic acid bacteria: classification and physiology. In: Lactic acid bacteria. Microbiology and functional aspects. (Salminen,S. and von Wright, A., Eds.), 2nd ed. pp. 369-383. Marcel Dekker Inc. New York,

[2]. Vaughan, E.E., de Vries, M.C., Zoetendal, E.G., Ben-Amor, K., Akkermans, A.D. and de Vos, V.M. (2002) The intestinal LABs. Antonie van Leeuwenhoek, 82, 341-352.

[3]. Smith, H.W. (1965) Observations on the flora of the alimentary tract of animals and factors affecting its composition. J Path. Bact. 89, 95-122.

[4]. Clapper, W.E., (1970) Microbiology. Gastrointestinal tract. In: The beagle as an experimental dog. (Anderson, A.C. and Good, L.S., Eds.) pp. 469-473. Ames Ia.: Iowa State University.

[5]. Davis, C.P., Cleven, D., Balish, E. and Yale, C.E. (1977) Bacterial association in the gastrointestinal tract of beagle dogs. Appl. Environ. Microbiol. 34, 194-206.

[6]. Benno, Y., Nakao, H., Uchida, K. and Mitsuoka, T. (1992) Individual and seasonal variations in the composition of fecal microflora of beagle dogs. Bifidobact. Microfl. 11, 69-76.

[7]. Greetham, H.L., Giffard, C., Hutson, R.A., Collins. M.D. and Gibson, G.R. (2002) Bacteriology of the Labrador dog gut: a cultural and genotypic approach. J. Appl. Microbiol. 93, 640-646.

[8]. Wilsson-Rahmberg, M. and Jonsson, O. (1997) Method for long-term intestinal acces in the dog. Laboratory Animals 31, 231-240,

[9]. Pitcher, D. G., Saunders, N. A. and Owen, R. J. (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. 8, 151-156.

[10]. Björkroth, J. and Korkeala, H. (1996) Evaluation of *Lactobacillus sake* contamination in vacuum-packaged sliced cooked meat products by ribotyping. J. Food Protect. 59**,** 398-401.

[11]. Grimont, F. and Grimont, P. A. D. (1986) Ribosomal ribonucleic acid gene restriction as potential taxonomic tools. Ann. Inst. Pasteur/Microbiol. 137B, 165-175.

[12]. Blumberg, H.M., Kielbauch, J.A. and Wachsmuth, K. (1991) Molecular epidemiology of *Yersinia enterocolitica* O:3 infection: use of chromosomal DNA restriction fragment length polymorphism of rRNA gene. J. Clin. Microbiol. 20, 2368-2374.

[13]. Farrow, J.A.E., Kruze, J., Philips, B.A., Bramley, A. J. and Collins, M.D. (1984) Taxonomic studies on *Streptococcus bovis* and *Streptococcus equinus*:

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

[14]. Baele, M., Devriese, L.A., Butaye, P. and Haesebrouck, F. (2002) Composition of enterococcal and streptococcal flora from pigeon intestines. J. Appl. Microbiol. 92, 348-351.

[15]. Robinson, I.M., Stromley, J.M., Varel, V.H and Cato, E.P. (1988) *Streptococcus intestinalis*, a new species from the colons and feces of pigs. Int. J. Syst. Bacteriol. 38, 245-248.

[16]. Vandamme P., Devriese L.A, Haesebrouck F. and Kersters K. (1999) *Streptococcus intestinalis* Robinson et al. 1988 and *Streptococcus alactolyticus* Farrow et al. 1984 are phenotypically indistinguishable. Int J Syst Bacteriol. 49, 737-741.

[17]. Salminen, S. and Deighton, M. (1992) Lactic acid bacteria in the gut in normal and disordered states. Dig. Dis.10, 227-238.

[18]. Hudault S, Lievin, V., Bernet-Camard, M.F. and Servin, A.L. (1997) Antagonistic activity exerted in vitro and in vivo by *Lactobacillus casei* (strain GG) against *Salmonella typhimurium* C5 infection. Appl. Environ. Microbiol. 63, 513-518.

[19]. Pascual M., Hugas M., Badiola J.I., Monfort J.M. and Garriga M. (1999) *Lactobacillus salivarius* CTC2197 prevents *Salmonella enteritidis* colonization in chickens. Appl. Environ. Microbiol. 65, 4981-4986.

[20]. Gill, H.S., Rutherfurd, K.J., Prasad, J. and Gopal, P.K. (2000) Enhancement of natural and aquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). Br. J. Nutr. 83, 167-176.

[21]. Vitini, E., Alvarez, S., Medina, M., Medici, M., de Budeguer, M.V. and Perdigon, G. (2000) Gut mucosal immunostimulation by lactic acid bacteria. Biocell. 24, 223-232.

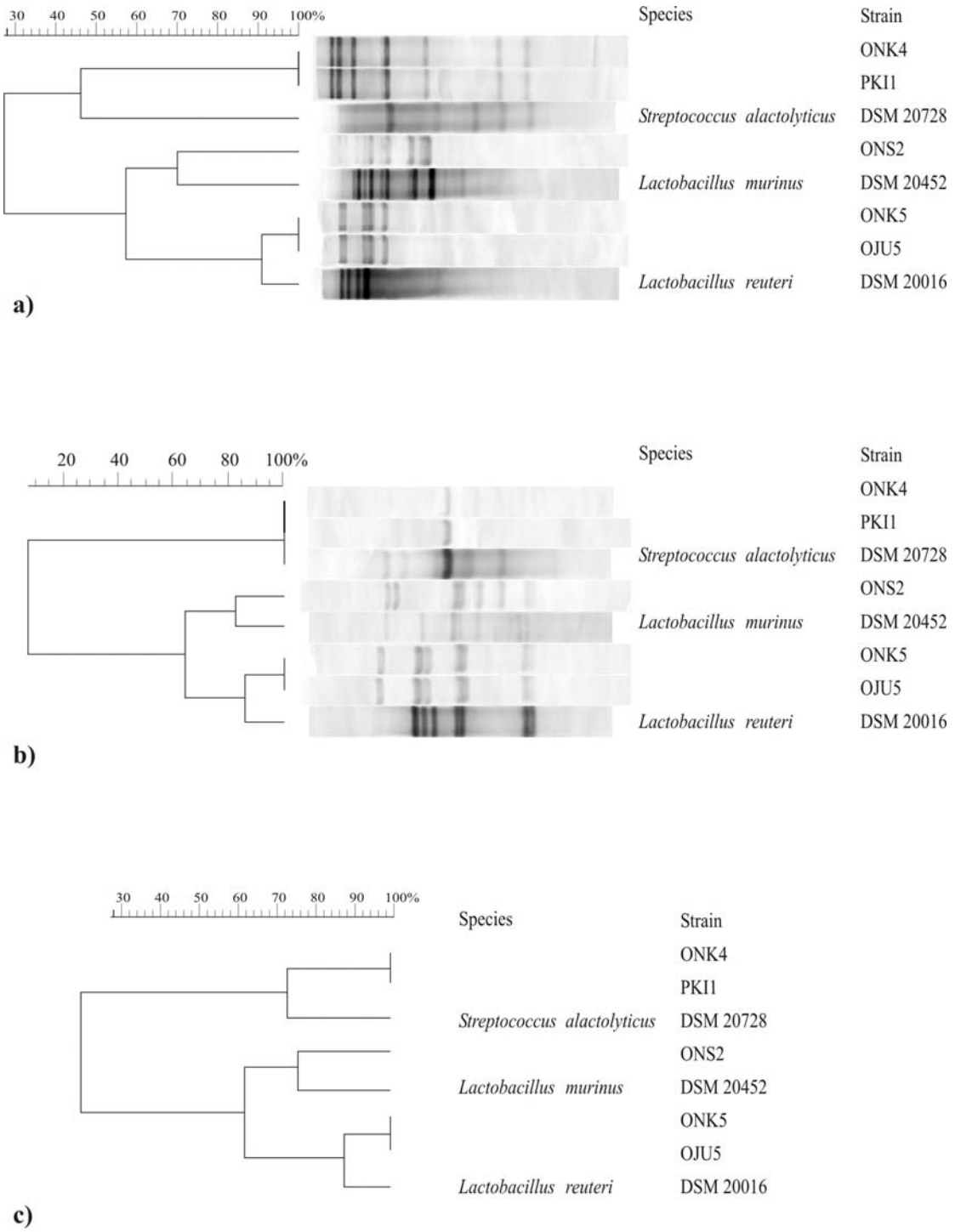
309 Table 1. Species division (number of isolates) within the LAB 30 isolates cultured
310 pure from jejunal chyme or feces of 4 castrated male dogs with permanent jejunum
311 nipple valve fistulas. Species were identified by the means of a RFLP database and
312 16S rDNA sequencing.
313

	Dog 1		Dog 2		Dog 3	Dog 4
LAB species	jejunal chyme	feces	jejunal chyme	feces	jejunal chyme	jejunal chyme
Streptococcus alactolyticus	4	5	3	3	2	3
Lactobacillus reuteri					2	2
Lactobacillus murinus	1		2	2	1	

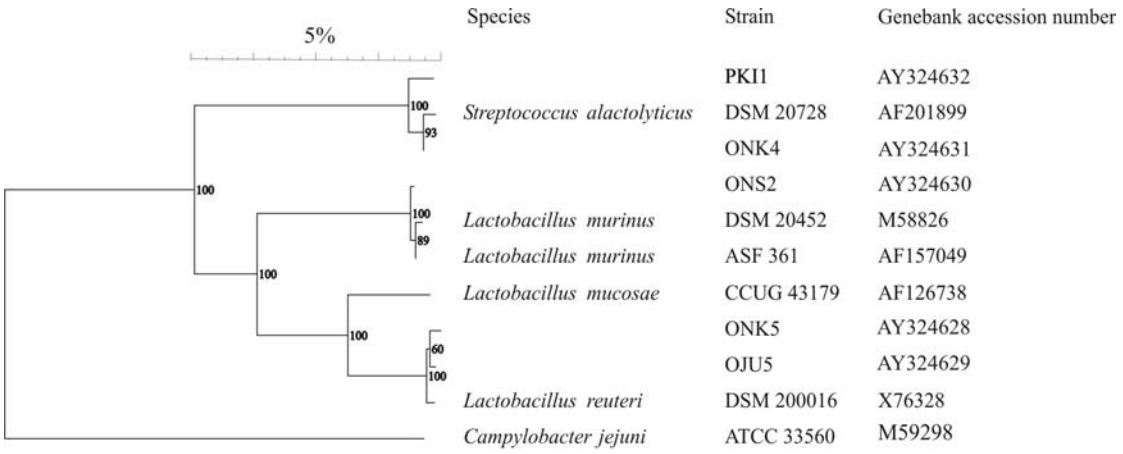
314

Fig. 1. (a), (b) and (c) present numerical analysis of 16 and 23S RFLP patterns (ribotypes) generated by *Eco*RI, *Hin*dIII 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 *Eco*RI and *Hin*dIII 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.



328 Fig. 1, Rinkinen et al.



329 Fig. 2, Rinkinen et al.
330