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Lactobacillus agilis is an important component of the pigeon crop flora

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Aims: To examine the presence of lactic acid bacteria (LAB: enterococci, streptococci and lactobacilli) in the pigeon crop.

Methods and Results: The crops of 10 pigeons were sampled and inoculated on agar plates for isolation of streptococci, enterococci and lactobacilli. The isolates were identified using tDNA-PCR. *Lactobacillus agilis*, a species described in 1981 from municipal sewage, was the dominant component in eight of these pigeon crop sacs. A *Lactobacillus* species related to *L. fermentum* and *L. mucosae* but probably not belonging to one of these species was isolated from five birds. Three pigeons carried *Enterococcus cecorum*. Minor species found were *E. columbae*, *E. faecalis*, *E. hirae*, *L. johnsonii*, *L. salivarius*, and *Streptococcus gallolyticus*. A description is given of the phenotypic characteristics of the *L. agilis* pigeon strains.

Conclusions, Significance and Impact of the Study: *L. agilis* is found to be the main component of the LAB flora in the pigeon crop.

INTRODUCTION

The pigeon crop flora has only vaguely been described as being composed mainly of lactobacilli and streptococci (Shetty *et al.* 1990). These bacteria never have been identified to species. In this study, an attempt was made to further investigate the crop flora in pigeons for the presence of enterococci, streptococci and lactobacilli. Isolates were identified to the species level using tDNA-PCR, a technique described first by Welsh and McClelland (1991). In our study, it has been applied in combination with capillary electrophoresis as described previously (Vaneechoutte *et al.* 1998; Baele *et al.* 2000).

As it was found that the main component of the pigeon crop flora was *Lactobacillus agilis*, a poorly known species (Weiss *et al.* 1981) which has been described only from a different, but probably related source (municipal sewage), a more extensive investigation was undertaken to characterize these strains.

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MATERIALS AND METHODS

Isolations

Crop sac samples of 10 euthanized pigeons from as many different lofts were taken. The pigeons that had been submitted for necropsy because of suboptimal performance were found to be infected by parasites associated commonly with pigeons or had mild respiratory infections of unknown aetiology. The samples were inoculated on Rogosa SL agar (Becton-Dickinson, USA) for the selective isolation of lactobacilli, and on CNA blood agar (Columbia agar base obtained from Oxoid, Basingstoke, UK, supplemented with colistine, nalidixic acid) with 50% ovine blood and on Slanetz & Bartley agar (Oxoid) to select other Gram-positive bacteria. Rogosa plates were incubated anaerobically and CNA agar plates in 5% CO_2 for two d. Colonies representing all discernible growth types (one representative for each) were purified, Gram-stained and stored frozen at −20°C.

DNA preparation

Bacterial cells were grown overnight on Columbia agar (Gibco Technologies, Paisley, Scotland, UK) with 5% ovine blood or on Rogosa agar for 24 h at 37°C in a 5% CO_2-enriched environment and checked for purity. A small loopful of cells

was suspended in 20 μl lysis buffer (0·25% SDS, 0·05 N NaOH) and heated at 95°C for 5 min. After brief centrifugation at 16000 *g*, lysed cells were resuspended in 180 μl distilled water and centrifuged again for 5 min at 16000 *g* to remove the cell debris. Supernatants were used as the DNA in the PCR or were frozen at –20°C until further use.

tDNA-intergenic PCR (tDNA-PCR)

PCR was carried out using outwardly directed tRNA-gene consensus primers T5A (5′AGTCCGGTGCTCTAACCA-ACTGAG) and T3B (5′AGGTCGCGGGTTCGAATCC) as described earlier (Welsh and McClelland 1991). Cycle reactions were carried out as previously described (Baele *et al*. 2000).

Capillary electrophoresis

One microlitre of tDNA-PCR product was mixed with 0·5 μl of an internal size standard mixture, containing 0·3 μl of the GS-400 high density size standard and 0·2 μl of the GS-500 size standard, both containing fluorescently (ROX) labelled fragments in the range of 50–500 bp. The mixtures were denatured in 12 μl de-ionized formamide by heating at 95°C for 3 min and placed directly on ice for at least 15 min (according to the recommendations of the manufacturer). Capillary electrophoresis was carried out using an ABI-Prism^{TM} 310 Genetic Analyser (Applied Biosystems, CA, USA) at 60°C, with a constant voltage of 1·5 kV, 10 mA. Electropherograms were normalized using the internal size standards with Genescan Analysis software, version 2·1.

Data analysis

Electropherograms were interpreted with a software program developed at our laboratory (Baele *et al*. 2000).

Sequencing

16S-rDNA was amplified using the commercially available Qiagen taq mastermix, to which primers $\alpha\beta$-not (5′TCAAACTAGGACCGAGTC) and ω_{MB} (5′TAC-CTTGTTACTTCACCCCA) were added at a concentration of 0·2 μM. Template DNA was added in a 1/10 dilution. After 5 min of denaturation at 95°C, reaction mixtures were cycled three times in a Perkin-Elmer Cetus 9600 thermocycler under the following conditions: 45 s at 95°C, 2 min at 55°C and 1 min at 72°C, and 30 times under the following conditions: 20 s at 95°C, 1 min at 55°C and 1 min at 72°C. After final extension for 7 min at 72°C, the reaction mixtures were cooled to 10°C. Before performing the sequencing reaction, the amplification products were purified from primers and nucleotides using the PCR product presequencing kit (Amersham-Pharmacia Biotech, Roosendaal, The Netherlands), which contains the enzymes shrimp alkaline phosphatase and exonuclease I. The PCR products were sequenced using the bigdye terminator sequencing kit (Applied Biosystems) and primers pD, gamma*, 3 and O* (Coenye *et al*. 1999) and determined on an automatic DNA sequencer (Abi Prism^{TM} 310 Genetic Analyser; Applied Biosystems). The electropherograms were exported and converted to genebase (Applied Maths, Kortrijk, Belgium) using ABICONV (Applied Maths). The sequences were compared to the NCBI genbank using the blast search tool.

Biochemical activity and growth characteristics

Eight strains, each representing a different loft, identified as *L. agilis* by tDNA–PCR were tested biochemically and in different growth conditions and compared with the *L. agilis* type strain LMG 9186^{T} obtained from the LGM collection (Laboratorium voor Microbiologie, Ledeganckstraat 35, B9000 Ghent, Belgium) as described earlier (Devriese *et al*. 1993, 1998).

RESULTS

Forty-two isolates were subjected to tDNA-PCR analysis. Eighteen of these proved to be *L. agilis*. Their tDNA pattern consisted of two peaks representing fragments of 150 bp and 172 bp and was identical to the patterns of the *L. agilis* type strain LMG 9186^{T} and two other collection strains, LMG 11398 and LMG 13085. The identification was confirmed by sequencing of the 16S rDNA gene of one of these strains. Eight of the 10 crop samples examined were positive for this species. They accounted for the most common colony types on Slanetz and Bartley medium (39%), as well as on Rogosa (58%). Two colonies from Rogosa medium originating from two pigeons were identified as *L. salivarius,* and one was *L. johnsonii*. Seven colonies from five birds could not be identified with any of the 42 known *Lactobacillus* species in the database. Based on the percentages of homology in 16S rRNA sequences their closest relatives appeared to be *L. mucosae* (95·9%) and *L. fermentum* (95·5%). The 16S rDNA sequence of one of these strains has been submitted to GenBank with accession number AF333975.

Three colonies growing on CNA blood agar from three pigeons were identified as *Enterococcus cecorum*, and two others from the same medium proved to be *E. columbae*. One colony was identified as *Streptococcus gallolyticus*. Five isolates representing different colony types growing on CNA blood agar could not be identied as one of the 212 Gram-positive species included in the tDNA-PCR database. All five showed tDNA-electropherograms differing from

Table 1 Variable carbohydrate acidification reactions of *L. agilis* strains from pigeons ($N = 8$) and the *L. agilis* type strain as determined in this study, and reactions differing from the currently available descriptions based on strains from sewage (Weiss *et al*. 1981; Hammes and Vogel 1995)

Characteristic	Pigeon strains	Type strain	Sewage strains
N-acetyl-glucosamine	7	+	ND
Amygdalin	7	+	+
L-arabinose	1	–	–
Melezitose	2	–	+
Mannitol	3	+	+
Raffinose	4	+	+
Sorbitol	0	–	V
D-turanose	1	–	ND
D-xylose	1 weak	–	+

ND: not determined V: variable.

each other as well. These strains were not investigated in more detail.

The eight *L. agilis* strains investigated phenotypically did grow on blood containing media to very small non-haemolytic or α-haemolytic colonies. Their colony sizes were larger when incubated in 5% CO_2 in air or anaerobically in a $H_2 + CO_2$ atmosphere but they did not exceed 1 mm. On Rogosa medium they were larger and most often slightly rough. Growth on this medium was also improved when incubated in 5% CO_2, and even more by anaerobic incubation. They were unable to grow at 25°C and grew equally well at 37°C as at 42°C. All strains produced acid from arbutin, cellobiose, D-fructose, galactose, β-gentiobiose, D- glucose, lactose, maltose, D-mannose, melibiose, ribose (some only weakly), saccharose, salicin and trehalose. All strains splitted esculin. Variable reactions and test results differing from the *L. agilis* descriptions available in the literature are listed in Table 1.

DISCUSSION

In this study *L. agilis* was found to be the main component of the pigeon crop lactic acid bacteria flora. Most probably the *L. agilis* strains possess specific attachment mechanisms to crop epithelium, as has been documented amply in the case of the chicken crop and other lactobacilli (Fuller 1978). It is extremely possible that the origin of the strains from municipal sewage on which the original description of this species was based (Weiss *et al*. 1981) is to be searched for in pigeons, and that the crop of pigeons and possibly other birds is the real habitat of this poorly known species. The inability of *L. agilis* to grow at 25°C is a strong argument in favour of this hypothesis. No other isolations have been reported after the first description of these bacteria. Despite this apparent association with a single host species, *L. agilis* has been shown to be able to maintain itself in humans up to 11 d following ingestion (Johansson *et al*. 1993). The carbohydrate acidification reactions of the pigeon strains appeared to be more variable than those of the sewage strains (Table 1), but no differences were observed between these two groups.

The other bacteria identified were less frequent. The determination of the exact taxonomic position of the strains related to *L. fermentum* and *L. mucosae* isolated in the present study requires more extensive investigation and falls outside the scope of the present communication. The presence of *E. columbae* and *S. gallolyticus* in the pigeon intestinal tract was to be expected. The former species has been described from pigeons (Devriese *et al*. 1990) and it has been found to date only in this animal host. *S. gallolyticus* is an opportunistic pigeon pathogen (Devriese *et al*. 1998), which was formerly indiscernible from *S. bovis* (Devriese *et al*. 1990). *E. cecorum* is associated with a wide variety of animal species. This *Enterococcus* is difficult to differentiate from the phylogenetically related *E. columbae* (Devriese *et al*. 1993).

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