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NOTE

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Lactobacillus colini sp. nov., isolated from Northern Bobwhite (*Colinus virginianus*)

Michael Z. Zhang,[1] Ming Yang,[2] Hongwen Su,[3] Dale Rollins[4] and Shuping Zhang[2,*]

Abstract

Biochemical and molecular studies were performed on five unknown bacterial strains isolated from the intestinal contents of Northern Bobwhites (*Colinus virginianus*) collected from western Texas, USA. The strains were Gram-stain-positive, catalase-negative, non-spore-forming rods arranged in single cells, pairs or short chains. Colonies on Columbia blood agar are circular, flat, entire, approximately 0.5–1.5 mm in diameter and surrounded with a zone of alpha-haemolysis at after incubation for 48 h at 37 °C. Colonies on MRS agar are umbonate with irregular edge, opaque and approximately 1–1.5 mm in diameter after incubation for 48 h. The 16S rRNA gene sequences of the isolates were identical and the highest sequence similarity (97 %) was found to the type strains of *Lactobacillus gasseri*, *L. johnsonii* and *L. taiwanensis*. The strains were distinguishable from related species of the genus *Lactobacillus*on the basis of carbohydrate fermentation, enzymatic production and fatty acid profiles. The peptidoglycan type is L-Lys-D-Asp (A4α). The DNA G+C content is 35.6 mol%. Major cellular fatty acids are $C_{14:0}$, $C_{16:0}$ and $C_{18:1}\,\omega9c$. Based on phenotypic, phylogenetic and chemotaxonomic information, the strains represent a novel species of the genus *Lactobacillus* for which the name *Lactobacillus colini* sp. nov. is proposed. The type strain is 111144 L1^T (=DSM 101872^T=KCTC 21086^T).

Species of the genus *Lactobacillus* were first described in 1901 [1] and at the time of writing, the genus comprises more than 250 species and subspecies with validly published names according to LPSN (http://www.bacterio.net/index.html). Members of the genus *Lactobacillus* are Gram-stain-positive, non-spore-forming, catalase-negative bacilli and coccobacilli. Owing to their metabolic properties, some species of the genus *Lactobacillus* are used as probiotics, vaccine carriers and starter cultures for controlled fermentation [2–4]. Species of the genus *Lactobacillus* are widely distributed in nature and routinely isolated from the intestinal tract and mucosal membrane of humans and animals as well as foods and man-made habitats. Northern Bobwhite belongs to the order Galliformes which consists of ground-feeding birds such as chicken, turkey, and grouse. A number of species of the genus *Lactobacillus* have been isolated from the intestinal tract of chickens, including *Lactobacillus kitasatonis*, *Lactobacillus gigeriorum* and '*Lactobacillus alvi*' [5–7]. During an investigation into the possible cause(s) of a declining bobwhite population, cultivable intestinal microbiota of wild-caught bobwhites was characterized [8]. None of the quail used in the study displayed any clinical signs of disease or intestinal pathology during post-mortem examination. For bacterial culture, intestinal contents were inoculated onto Columbia blood agar with 5 % sheep blood, MacConkey agar and CDC anaerobe 5 % sheep blood agar with phenylethyl alcohol (PEA) (Thermo Fisher Scientific Remel Products). Inoculated Columbia blood agar plates were incubated at 37 °C in a 5 % CO_2 atmosphere, MacConkey agar plates were incubated at 37 °C without CO_2, and PEA agar plates were incubated at 37 °C in an AnaeroPack jar with an AnaeroPouch (Thermo Fisher Scientific Remel Products).

After incubation for 24 h, moderate growth of pinpoint colonies was observed on Columbia and anaerobic PEA agar plates. At 48 h, colonies were small (approx. 0.5–1.5 mm in diameter), grey, circular, flat with raised centre and surrounded by a wide zone of partial haemolysis. Single colonies on Columbia blood agar and anaerobic PEA plates were subcultured onto fresh Columbia and MRS agar plates (Thermo Fisher Scientific) for purity. The subcultures were incubated at 37 °C in 5 % CO_2 for 48 h prior to identification. Bacterial

Author affiliations: [1]Department of Biomedical Sciences, College of Veterinary Medicine, University of Missouri, Columbia, MO 65211, USA; [2]Department of Veterinary Pathobiology, Veterinary Medical Diagnostic Laboratory, College of Veterinary Medicine, University of Missouri, Columbia, MO 65211, USA; [3]Department of Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77845, USA; [4]Rolling Plains Quail Research Foundation, Texas AgriLife Research, San Angelo, TX 76901, USA.
***Correspondence:** Shuping Zhang, zhangshup@missouri.edu
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The GenBank/EMBL/DDBJ accession numbers for the 16s rRNA gene, *groEL* and *rpoB* sequences of strain 111144 L1^T are KU161105, KU850952 and KU850953, respectively.
Two supplementary figures are available with the online Supplementary Material.

identification by Bruker Biotyper matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer and API 50 CHL biochemistry strip (bioMérieux) failed to assign the isolates to any known species. Gram stain and Schaeffer–Fulton stain (Sigma-Aldrich) revealed Gram–stain-positive, non-spore-forming rods arranged in single cells, pairs or short chains. The isolates were oxidase- and catalase-negative. Growth characteristics of five isolates were evaluated by incubating in BHI and MRS broth for 7 days at various temperatures (4, 10, 15, 20, 25, 30, 37, 40, 45 and 50 °C), pH conditions (pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 10) and sodium chloride concentrations (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 %, w/v). Bacterial growth occurred at temperatures between 25 and 45 °C and pH between 3.5 and 10. The isolates tolerated 3 % NaCl. Optimal growth was observed at 37 °C, pH 5 and less than 1 % NaCl. Phase contrast microscopic examination of 10 h MRS and BHI cultures indicated that the organisms were non-motile.

DNA was prepared using the DNeasy Blood & Tissue Kit (Qiagen). The complete 16S rRNA gene sequences (1542 bp) were determined as described previously [9]. GenBank BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) search revealed that the five isolates were identical and shared 97 % 16S rRNA gene sequence similarity with *Lactobacillus gasseri*, *Lactobacillus johnsonii* and *Lactobacillus taiwanensis*. In contrast, the 16S rRNA gene sequence similarities between the five isolates and species of the genus *Lactobacillus* from chickens (*L. kitasatonis*, *L. gigeriorum* and '*L. alvi*') were less than 95 % [5–7]. Multiple alignments of the 16S rRNA gene sequences (1500 bp) were conducted using CLUSTAL W and phylogenetic reconstruction was performed using the neighbour-joining method [10–13]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Fig. 1). The five quail isolates formed one cluster that was closely related to *L. gasseri* ATCC 33323^T, *L. johnsonii* ATCC 33200^T and *L. taiwanensis* BCRC 17755^T (Fig. 1). In addition, the sequences of two housekeeping genes, *groEL* and *rpoB* encoding heat-shock protein 60 and the β-subunit of RNA polymerase, respectively, were also determined as described previously [14, 15]. Phylogenetic trees based on *groEL* sequences (470 bp) and *rpoB* sequences (310 bp)

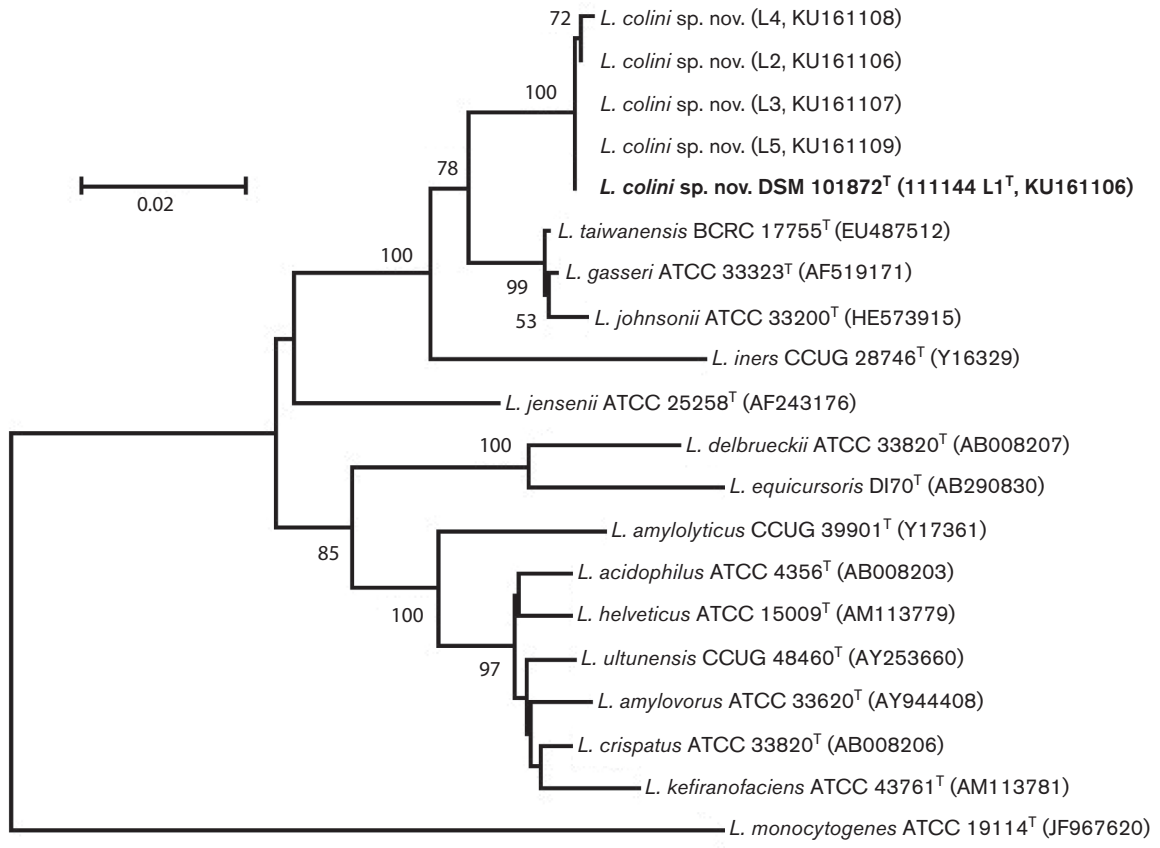

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1500 bp) showing the relationship of the strains isolated from Bobwhite quail intestinal content with other species of the genus *Lactobacillus*. The strains form a district group closely related to *L. gasseri*, *L. johnsonii* and *L. taiwanensis*. Numbers at the nodes represent bootstrap values ≥50 % (based on 1000 replications). *Listeria monocytogenes* ATCC 19114 was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

supported the relationship with the *L. gasseri* group (Figs S1 and S2, available in the online Supplementary Material). While *L. gasseri* and *L. johnsonii* are well known for their probiotic properties, *L. taiwanensis* was isolated from a silage [16–18]. The results of comparative 16S rRNA gene sequence analysis indicated that the five isolates represent a novel species of the genus *Lactobacillus* [19]. To reflect the origin of the isolates, the name *Lactobacillus colini* sp. nov. was proposed and isolate 11144 L1^T was designated as the type strain.

Carbohydrate fermentation patterns and enzymatic activities of all five isolates were examined using API 50 CHL and API ZYM test strips (bioMérieux), respectively. *L. gasseri* ATCC 33323^T was included as a reference species. The biochemical characteristics that differentiate *L. colini* sp. nov. from its nearest phylogenetic neighbours are summarized in Table 1.

Additionally, the cellular fatty acid profiles of *L. colini* sp. nov and closely related species of the genus *Lactobacillus* were determined by gas chromatography using the Agilent ChemStation and Sherlock software (Microbial ID). *L. colini* sp. nov. 111144 L1^T could be differentiated from other closely related species based on the cellular fatty acids profile (Table 2). The major fatty acids of strain 111144 L1^T were $C_{14:0}$, $C_{16:0}$ and $C_{18:1}\omega9c$ whereas *L. gasseri* ATCC 33323^T, *L. johnsonii* ATCC 33200^T and *L. taiwanensis* DSM 21401^T contained $C_{17:0}$ and $C_{18:1}\omega9c$; $C_{16:0}$, $C_{18:1}\omega9c$ and C_{19} cycloprop. 9, 10; $C_{16:0}$, $C_{18:1}\omega9c$, C_{19} cycloprop. 9, 10 and C_{19} cycloprop. 11, 12; respectively, as their major fatty acids.

The cell-wall peptidoglycan structure was determined by the Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) according to a previously published method [20]. The cross-linkage type for *L. colini* sp. nov. 111144 L1^T is L-Lys-D-Asp (A4α). This type of peptidoglycan cross-linkage is found in the majority of known species of lactobacilli [21]. The DNA G+C content was analysed by HPLC at the DSMZ as described previously [22, 23]. In brief, DNA was hydrolysed with P1 nuclease and nucleotides dephosphorylated as described previously [23]. The resulting deoxyribonucleosides were analysed by HPLC and the G+C value was calculated according to Mesbah *et al.* [23]. The G+C content of *L. colini* sp. nov. 111144 L1^T is 35.6 mol%.

In summary, the results of carbohydrate fermentation, fatty acid composition, peptidoglycan cross-linkage and DNA G+C content further confirmed that the bacterial isolates under investigation constitute a novel member of the genus *Lactobacillus*, for which the name *Lactobacillus colini* sp. nov. is proposed.

DESCRIPTION OF *LACTOBACILLUS COLINI* SP. NOV.

Lactobacillus colini (co.li′ni. N.L. gen. n. *colini* of *Colinus*, scientific name of bobwhites).

Cells are Gram-stain-positive, catalase-negative, non-spore-forming and non-motile rods. Cells are found singly, in pairs and in short chains. Facultatively anaerobic. Colonies on Columbia blood agar are circular, flat, entire,

Table 1. Characteristics that differentiate *Lactobacillus colini* sp. nov. from its nearest phylogenetic relatives

Taxa: 1, *L. colini* sp. nov. 111144 L1^T; 2, *L. colini* sp. nov. L2; 3, *L. colini* sp. nov. L3; 4, *L. colini* sp. nov. L4; 5, *L. colini* sp. nov. L5; 6, *L. gasseri* ATCC 33323^T; 7, *L. taiwanensis* CIP 110030^T; 8, *L. johnsonii* ATCC 33200^T. Data for taxa 1 to 6 are Data for *L. taiwanensis* and *L. johnsonii* were obtained from [18] and [24]. +, Positive; −, negative; NA, data not available.

Characteristic	1	2	3	4	5	6	7	8
Growth at/with:								
20 °C	−	−	−	−	−	−	−	+
4 % NaCl	−	−	−	−	−	+	+	+
Acid production from:								
N-Acetylglucosamine	+	+	+	+	+	+	−	+
Amygdalin	+	+	+	+	+	+	−	−
D-Ribose	+	+	+	−	+	−	−	−
D-Mannitol	+	+	+	+	+	−	−	−
Methyl α-D-Glucopyranoside-NAG	+	+	+	+	+	−	−	−
Lactose	−	−	−	−	−	+	+	+
Melezitose	+	+	+	+	+	−	−	−
Raffinose	+	+	−	+	+	−	−	+
Gentiobiose	−	−	−	+	−	−	+	+
D-Tagatose	−	−	−	−	−	+	−	+
Enzymatic activity								
Naphthol-AS-BI-phosphohydrolase	−	−	−	−	−	+	NA	+
DNA G+C content (mol%)	35.6	35.6	35.6	35.6	35.6	35.8	35.8	35.9

Table 2. Cellular fatty acid contents (%) of *Lactobacillus colini* sp. nov. and closely related species

Taxa: 1, *L. colini* sp. nov. 111144 L1^T; 2, *L. gasseri* ATCC 33323^T; 3, *L. johnsonii* ATCC 33200^T; 4, *L. taiwanensis* DSM 21401^T. All data are from this study. Fatty acids present at >10% are indicated in bold. –, Not detected.

Fatty acid	1	2	3	4
$C_{12:0}$	0.4	–	0.3	–
$C_{14:0}$	**12.0**	–	2.2	5.5
$C_{14:0}$ 3-OH	0.2	–	–	–
$C_{14:1}\omega5c$	–	–	0.5	–
anteiso-$C_{15:0}$	–	–	–	0.1
$C_{15:0}$	–	–	–	0.2
$C_{16:0}$	**33.9**	0.2	**10.3**	**24.2**
$C_{16:0}$ 3-OH	0.2	–	–	–
$C_{16:1}\omega7c$	1. 1	1.1	4.6	3.8
anteiso-$C_{17:0}$	–	–	0.3	0.3
$C_{17:0}$	0.2	**16.5**	–	–
$C_{17:0}$ 2-OH	1.2	2.4	–	–
$C_{17:1}\omega8c$	0.2	0.2	–	–
$C_{18:0}$	4.4	0.1	3.5	1.8
$C_{18:1}\omega7c$	7.4	5.6	–	–
$C_{18:2}\omega7c$	–	–	1.2	–
$C_{18:1}\omega9c$	**35.5**	**68.6**	**41.1**	**12.5**
iso-$C_{19:0}$	1.2	1.6	–	–
iso-$C_{19:1}$	0.4	0.4	–	–
C_{19} cycloprop. 9,10	–	–	**23.2**	**19.2**
C_{19} cycloprop. 11,12	–	–	–	**13.1**
$C_{20:0}$	1.8	2.2	–	–
$C_{20:4}\omega6,9,12,15c$	–	0.1	–	–
$C_{20:1}\omega7c$	–	0.1	–	–
Summed features*				
2	0.2	–	–	–
3	1.1	1.1	–	–
8	–	–	0.9	0.5
10	–	–	7.0	**13.8**

*Summed features are groups of two or more fatty acids that could not be separated by GC. Summed feature 2 contained $C_{12:0}$ aldehyde; summed feature 3 contained $C_{16:1}\omega7c/C_{16:1}\omega6c$; summed feature 8 contained $C_{17:1}\omega8c$; summed feature10 contained $C_{18:1}\omega7c$.

approximately 0.5–1.5 mm in diameter and surrounded with a zone of alpha-haemolysis after incubation for 48 h at 37 °C. Colonies on MRS agar are umbonate with irregular edge, opaque and approximately 1–1.5 mm in diameter after incubation for 48 h. Grows in BHI and MRS broth containing 0.5 to 3 % NaCl at temperatures between 25 and 45 °C and pH from 3.5 to 10. Optimal growth occurs at 37 °C, pH5 to 5.5 and with less than 1 % NaCl. Acid is produced from cellobiose, D-fructose, D-galactose, D-glucose, D-mannose, maltose, D-mannitol, melezitose, sucrose, salicin and trehalose. Hydrolyses amygdalin, arbutin, aesculin, methyl α-D-glucopyranoside NAG, *N*-acetylglucosamine and starch. Produces leucine arylamidase, α-galactosidase, α-glucosidase and β-glucosidase. The peptidoglycan type is L-Lys-D-Asp (A4α).

The type strain is 111144 L1^T (=DSM 101872^T=KCTC 21086^T), isolated from the intestine of a bobwhite quail. The G+C content of the type strain is 35.6 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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