Recognition of two subspecies of *Lactobacillus amylovorus*, with proposal of *Lactobacillus amylovorus* subsp. *animalis* subsp. nov. isolated from bovine feces and *Lactobacillus amylovorus* subsp. *amylovorus*, and an emended description of *Lactobacillus amylovorus*

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains BF125^T, BF186, YK3, YK6, YK10, and DSM 16698 are LC771959–LC771964. The INSDC accession numbers for the draft genome sequences of strains BF125^T, BF186, YK3, YK6, and YK10 are BTFR01000001-BTFR01000033, BTFQ01000001–BTFQ01000077, BTFS01000001–BTFS01000055, BTFT01000001–BTFT01000032 and BTFU01000001–BTFU01000072, respectively.

Five supplementary figures and two tables are available in the similar online version of this article.

ABSTRACT

Five novel lactic acid bacterial strains (BF125^T, BF186, YK3, YK6, and YK10) were isolated from the fresh feces of Japanese black beef cattle or spent mushroom substrates and characterized using a polyphasic taxonomy method. These strains are rod-shaped, Gram-stain-positive, nonmotile, non-spore-forming, catalase-negative, cytochrome oxidase-negative, facultatively anaerobic, and homofermentative. The cells of BF125^T were 0.5–0.7 μm in width and 3.0–7.0 μm in length. Strain BF125^T did not produce any gas from glucose; both D- and L-lactate were produced as end products of glucose (D/L, 40:60). Growth occurred at a temperature of 30–45°C (optimum, 37°C), pH of 5.0–8.0 (optimum, pH 6.0), and NaCl concentration of 1.0–3.0% (w/v). The GC content of genomic DNA of strain BF125^T was 37.8% (whole-genome analysis). The major fatty acids were $C_{16:0}$, $C_{18:1}$ ω9*c*, $C_{19:0}$ cyclo ω8*c,* and summed feature 10. Strain BF125^T retained high similarity of the 16S rRNA gene to the type strain of *Lactobacillus amylovorus* (99.93%), and the other isolates were also identified as *L. amylovorus* based on high 16S rRNA gene similarities. Comparison of the core genomes of *L. amylovorus* strains, including the five isolates, showed that they could be divided into two clusters, and phenotypic differences in fermentability were observed for D-lactose, salicin, and gentiobiose between these two groups. Digital DNA-DNA hybridization (dDDH) and average nucleotide identity (ANI) analyses showed that both categories were below the thresholds for defining subspecies (maximum dDDH value, 77.2%; maximum ANI value, 96.50%). In light of the physiological, genotypic, and phylogenetic evidence, we propose a novel subspecies of *L. amylovorus*, *L. amylovorus* subsp. *animalis* subsp. nov. Type strain: BF125^T (= MAFF 212522^T = DSM 115528^T). Our results also led to the automatic creation of *L. amylovorus* subsp. *amylovorus* subsp. nov. and an emended description of the species *L. amylovorus.*

Introduction

Lactic acid bacteria are a general term for bacteria that ferment sugars and produce large amounts of lactic acid [1]. They exist in various environments, including human and animal bodies, and have long been used in the production of fermented foods such as yogurt, cheese, pickles, and sake. Recently, as the useful functions of lactic acid bacteria (intestinal regulation, immunomodulation, prevention of arteriosclerosis, and antitumor action) have been scientifically proven, their effects on human health have attracted attention [2] .

Lactobacillus is the main genus of lactic acid bacteria, with 261 species being reported as of 2019 [3]. Their classification and identification are based on Gram staining, cell morphology, catalase reaction, lactic acid yield from glucose, endospore formation ability, and phylogenetic analyses via DNA-DNA hybridization (DDH) and 16S rRNA gene sequences. However, it has been pointed out that DDH is complicated [4] and that the high conservation of 16S rRNA gene sequences among bacterial species prevents adequate species inference for some groups of bacteria [5]. Therefore, with the development of next-generation sequencing in recent years, genome comparisons have been used for the classification and identification of bacterial species and subspecies, as well as for the identification of new taxa. In 2020, Zheng *et al*. conducted a genomic-level reclassification of all *Lactobacillus* species previously classified in this genus [3]. The analyses included average nucleotide identity (ANI), average amino acid identity, and core genome phylogeny. Based on these analyses, *Lactobacillus* was reclassified into 25 genera, and 23 new genera were proposed. After reclassification, we isolated *Lactobacillus corticis* B40^T as a novel species from woody biomass, a previously unreported source of

this genus [6]. At the time of writing, the genus *Lactobacillus* comprised 44 validly named species (www.bacterio.net; List of Prokaryotic names with Standing in Nomenclature) [7].

Lactobacillus amylovorus is a member of the genus (ex-) *Lactobacillus* and has been isolated from various environments, such as cattle waste-corn fermentation, the intestinal tracts of pigs and cattle, and corn steep liquor, a by-product of starch production from corn [8-11]. Certain *L. amylovorus* strains exhibit probiotic effects; for example, they can be used to improve lipid metabolism and as antibacterial agent [12, 13]. Research in the field of livestock production has also contributed to improvements in silage quality [14]. Thus, *L. amylovorus* is adaptable to a wide range of environments, including animals and plants, and is a promising lactic acid bacterium for commercial use (health, food, feed, and livestock production).

As part of a project that aims to isolate *L. amylovorus* strains with economically useful traits and elucidate their biodiversity and host adaptation, we isolated five *L. amylovorus* strains [two strains from bovine feces (BF125^T and BF186) and three strains from spent mushroom substrates (YK3, YK6, and YK10)] and characterized them using a polyphasic approach to determine their taxonomic position.

Isolation and ecology

Strains BF125^T and BF186 were isolated from fresh feces of Japanese black beef cattle reared in an experimental field of the Institute of Livestock and Grassland Science, NARO (Nasushiobara, Tochigi, Japan; longitude 139° 55′ 49″, latitude 36° 55′ 9″). Immediately after sampling, serial 10-fold dilutions of fecal homogenates were prepared with phosphate-buffered saline (pH 7.4). The diluted samples were streaked onto de Man, Rogosa, and Sharpe (MRS; Difco, Detroit, MI, USA) agar plates. After 3 d of incubation at 37 °C in an O_2-free environment containing $N_2/H_2/CO_2$ (80/10/10%) in an ANX-1 TE-HER Hard Anaerobox (Hirasawa, Japan), strains BF125^T and BF186 were isolated from fecal samples derived from different cattle. Strains YK3, YK6 and YK10 were isolated from a corn cob-based spent mushroom substrate collected from a temporary storage location in Hara-mura, Nagano, Japan (longitude 138° 16′ 7″, latitude 35° 58′ 45″) together with other strains that have been designated as novel species of the genera *Ligilactobacillus* and *Lentilactobacillus* [15, 16]. The three strains were isolated from the homogenates of the spent mushroom substrate following incubation on MRS agar plates for 72 h at 30 °C in the anaerobic chamber. The purified colonies were stored at −80 °C with 20% (v/v) dimethyl sulfoxide until analysis.

16S rRNA phylogeny

The 16S rRNA genes were amplified via PCR using primers 27F and 1492R [17] and sequenced as described previously [18]. The 16S rRNA sequences (nearly full-length) of all the isolates were compared with those of the type strains of the valid species using the EzBioCloud database [19]. The similarity values of the 16S rRNA gene sequences were calculated using a pairwise nucleotide sequence alignment [19]. Based on similarities in the 16S rRNA gene sequences, the two strains isolated from

bovine feces (BF125^T and BF186) were affiliated with the genus *Lactobacillus*, showing the highest sequence similarities with *L. amylovorus* DSM 20531^T (accession no. AZCM01000082; 99.93%), *L. kitasatonis* JCM 1039^T (BALU01000027; 99.60%), *L. crispatus* DSM 20584^T (AZCW01000112; 98.86%), and other *Lactobacillus* species (< 98.65% used for the threshold values for species differentiation) [20]. Three strains isolated from spent mushroom substrates also showed the highest similarity values of 100% (YK3), 99.93% (YK6), and 100% (YK10) to *L. amylovorus* DSM 20531^T. The sequences of the five isolates were 99.87–100% similar to each other.

The obtained 16S rRNA sequences were aligned with publicly available sequences of the type strains of closely related species and reference strains of *L. amylovorus* using MAFFT (version 7.520) [21] in auto mode. The multiple sequence alignment was trimmed by trimAl (version 1.4.1) with the parameter "-gappyout" [22]. A neighbor-joining (NJ) tree based on Kimura's two-parameter model [23] was reconstructed using RapidNJ (version 2.3.2) [24]. Maximum-likelihood (ML) analysis was performed in IQ-TREE (version 2.2.2.3) [25] using the best-fit model (TVMe+I+R2) selected using ModelFinder [26]. The alignment was also used to build a maximum-parsimony (MP) phylogenetic tree using MEGA software (version 11) [27]. The reliability of the tree topologies was evaluated using bootstrap analysis with 1,000 replications. The 16S rRNA phylogenetic trees obtained using the three algorithms were visualized using Interactive Tree Of Life (iTOL) (version 6) [28]. The overall NJ (Fig. S1), ML (Fig. S2), and MP (Fig. S3) topological structures revealed that the five isolates were in a clade containing reference strains and the type strain of *L. amylovorus*, suggesting that all isolates should be identified as *L. amylovorus*. Strains YK3, YK6, and YK10 belonged to an intraspecific group represented by *L. amylovorus* DSM 20531^T (group A), which was separated from another group, group B, which included strains BF125^T, BF186 and other references isolated from various animal origins, with strong bootstrap values (NJ 99%, ML 92%, and MP 99%). To clarify their taxonomic positions, a comparison based on whole genome sequences is presented in the following section.

Genome features

Genomic DNAs was extracted from strains BF125^T, BF186, YK3, YK6, and YK10 for whole-genome sequence analysis using Illumina MiSeq or NovaSeq 6000 (Illumina, San Diego, CA, USA), as previously reported [29, 30]. After filtering low-quality reads and trimming adapter sequences using fastp (version 0.23.4) [31], draft genomes were assembled using SKESA (version 2.4.0) [32] and annotated using the DFAST pipeline (version 1.2.0) [33]. Genomic quality was evaluated using the CheckM software (maker set, *Lactobacillus*) [34] in DFAST. Summary statistics for the genome sequences are shown in Table S1. After related draft genomes were obtained from the National Center for Biotechnology Information Assembly Database, the prediction and classification of clusters of orthologous groups (COGs) associated with the protein-coding sequences were analyzed using a Pan-genome Explorer [35] to obtain an initial view of the genetic diversity of *L. amylovorus*. The pan-genome shapes of the 10 analyzed *L. amylovorus* genomes are presented in Fig. S4. A total of 4,270 gene clusters (orthologs) were identified, of which 1,195 comprised the core genome (28.0%), 1,546 were dispensable genes (36.2%), and 1,529 were strain-specific genes (35.8%). The protein-coding genes of strain BF125^T were clustered into 18 COG categories, which were similar in all compared genomes, except for a minor COG category percentage of less than 1.2% (Fig. S5). The most abundant COG category in the genome of strain BF125^T was general function prediction only [R] (20.8%), followed by inorganic ion transport and metabolism [P] (15.3%), amino acid transport and metabolism [E] (12.3%), and carbohydrate transport and metabolism [G] (6.1%).

Phylogenetic analysis based on 166 conserved core genes was conducted using the PanACoTA pipeline (version 1.2.0) [36]. The ML core genome tree based on the best-fit model (GTR+F+I+R4) was reconstructed using IQ-TREE and ModelFinder and visualized using iTOL, as described above. The core genome phylogenetic tree revealed that the five isolates were assigned to *L. amylovorus*, and that the groups A and B formed distinct lineages within the same species, respectively, supported by 100% bootstrap value (Fig. 1). Similar to the results of 16S rRNA phylogeny, the type strain DSM 20531^T (isolated from cattle waste-corn fermentation [8]) and three strains isolated from spent mushroom substrates (YK3, YK6, and YK10 in the present study) were in the same cluster (group A), while group B included strains isolated from various animal origins; BF125^T (bovine feces, in the present study), BF186 (bovine feces, in the present study), Bifido-178-WT-3C (porcine feces, shown in the BioSample accession SAMN14558271), S60 (bovine nasopharynx [37]), AF08-3 (human feces, SAMN09734196), and DSM 16698 (intestine of weaning piglets [38]). Strain DSM 16698 was first proposed as a novel species in the genus ex-*Lactobacillus*, *Lactobacillus sobrius*, and is closely related to *L. amylovorus*. The original classification of the strain DSM 16698 was established by a low value of the classical DDH (49%), the ability to utilize raffinose and fructo-oligosaccharides, and the ability to grow at 45 °C as compared to the type strain of *L. amylovorus* [38]. However, a later study reclassified *L. sobrius* as a synonym of *L. amylovorus* based on the > 70% threshold value of the classical DDH experiments (> 79%) [39]. Our results and previous findings provide insights into the phenotypic and genotypic diversity of *L. amylovorus*.

To determine the exact taxonomic positions of the five isolates, ANI based on BLAST was calculated using JSpeciesWS [40], and digital DNA-DNA hybridization (dDDH) was performed using Genome-to-Genome Distance Calculator 3.0 [41]. Five isolates were identified as a single *L. amylovorus* species because they had ANI values greater than the 95–96% species boundary [42] against the type strain DSM 20531^T (Fig. 2). The ANI values within groups A and B ranged from 97.70–99.56% and from 97.58–99.96%, respectively, whereas the maximum ANI value between the groups was 96.50%. The ANI value between strain BF125^T (representative of group B) and the type strain DSM 20531^T (group A) was 96.21%. In addition to the proposed cut-off value for ANI to separate a prokaryotic subspecies (≥ 98%) [43], Tanizawa *et al*. evaluated ANI values among six subspecies of *Lactobacillus delbrueckii*, which is a type species of the genus *Lactobacillus*, and found that they ranged from 97.2–98.4% [44]. Hence, the ANI values between groups A and B were lower than those observed among *L. delbrueckii* subspecies, indicating that the two groups differ at the subspecies level. Furthermore, the highest dDDH value between the two groups was 77.2%, which was found to be below the threshold for recognition as a subspecies (79–80%) [45]. The dDDH value between strain BF125^T and the type strain DSM 20531^T was 74.1%, strongly suggesting that both groups are two novel subspecies.

Physiology and chemotaxonomy

Cell morphology was observed after 2 d of incubation at 37 °C under anaerobic conditions (AnaeroPack^{TM}-Anaero; Mitsubishi Gas Chemical Company, Japan). Gram staining was performed using a Gram Stain Kit (ScyTek Laboratories, Inc., UT, USA). Spore formation was confirmed using the Scheffer-Fulton-modified Wirtz staining method (Wirtz Stain Kit; Muto Pure Chemicals, Ltd., Tokyo, Japan). Colony morphology and size were observed after incubation on MRS agar plates for 2 d at 37 °C under anaerobic conditions. The presence of gas produced from the glucose in the MRS medium was determined using Durham tubes. Dextran production was observed in MRS agar medium

with sucrose (5%) replacing glucose as the carbon source, and colony status was observed. Catalase activity was determined by adding 3% (v/v) H_2O_2 to the colonies on the plates. The oxidase activity was determined using a cytochrome oxidase test strip '*Nissui*' (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. The lactic acid configuration was analyzed via an enzymatic method using an F-kit D-lactic acid/L-lactic acid kit (JK International, Inc., TN, USA). After the cells were incubated statically for 48 h in MRS broth at various pH (pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0), NaCl concentrations [0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10% (w/v)], or temperature conditions (15, 20, 25, 30, 37, 45 and 50 °C), the optical density at 660 nm and the culture pH were measured to determine pH tolerance, salt tolerance, and temperature growth. Physiological and biochemical characteristics were analyzed using API 50 CH strips (bioMérieux, France) and API ZYM (bioMérieux) in duplicate at 37 °C, according to the manufacturer's instructions. The cellular fatty acid profiles derived from cells cultivated on MRS agar plates at 37 °C for 2 d were analyzed using the Sherlock Microbial Identification System (MIDI) (version 6.0; database: MOORE6) by the identification service of Techno Suruga Laboratories.

Although the fatty acid compositions of the strains BF125^T, DSM 16698, and YK10 were relatively similar, differences were evident in strain DSM 20531^T, with $C_{14:0}$ being the major component. However, no particular fatty acid was a taxonomically useful marker for intraspecific differentiation (Table S2). The major fatty acids detected in strain BF125^T were $C_{18:1}$ ω9*c* (31.3%), $C_{16:0}$ (22.7%), C_{19} cyclo 9,10 (16.7%), and summed feature 10 (15.0%). In addition to genotypic evidence, the phenotypic differences between the two groups are summarized in Table 1. All tested strains in group A were able to ferment salicin and gentiobiose, whereas those in group B could not. Members of group B differed uniquely from those of group A in terms of being positive for acid production from lactose. Thus, groups A and B can be differentially characterized by their fermentability. Salicin is a β-glycoside found in several species of *Salix* and *Populus*, and β-glycoside is originally known in nature as a plant metabolite and is abundant in plant bodies [46]. Gentiobiose is a β-linked glucobiose found in gentian root [47], indicating that both salicin and gentiobiose are highly related to plants. Considering that strains of group A are isolated from plant-related sources, the ability to utilize these plant-related carbohydrates could play a significant role in the environmental adaptation of group A. In contrast, lactose, which is fermentable only by group B, is the major carbohydrate in most eutherian milks [48]. Members of Group B were isolated from various sources related to animals in the Eutheria group, as described above, suggesting that this group is more likely to be related to animals. Interestingly, analysis using the Pan-genome Explorer showed that group B retained a dispensable gene for lactose permease, which is an integral membrane protein responsible for lactose uptake [49], whereas group A did not. These results suggest that the mechanism underlying lactose uptake is an important factor in the diversity and host adaptation of *L. amylovorus*. Several variable reactions in each group were observed as follows; (i) D-mannitol, methyl-α-D-glucopyranoside, D-melibiose, D-raffinose, glycogen, and D-turanose assimilation in group A; (ii) D-galactose, D-mannose, and D-raffinose assimilation in group B; (iii) production of cystine arylamidase, α-galactosidase, and β-galactosidase in group A; and (iv) production of esterase lipase (C8) and α-galactosidase in group B. These findings suggest that these features are strain-specific and that all isolates obtained in this study are not clonal. Detailed physiological and biochemical characteristics are provided in the taxonomic descriptions below.

Based on the results of this polyphasic approach, we propose that *L. amylovorus* can be divided into two subspecies. We propose the creation of *Lactobacillus amylovorus* subsp. *animalis* subsp. nov. with the type strain BF125^T (= MAFF 212522^T = DSM 115528^T), and the automatic creation of *Lactobacillus amylovorus* subsp. *amylovorus* subsp. nov. with the type strain (ATCC 33620^T = CCUG 27201^T =CIP 102989^T = DSM 20531^T =JCM 1126^T = LMG 9496^T = NCAIM B.01458^T = NRRL B-4540^T). Based on the results of the newly reported characteristics in the present study, an emended description of *Lactobacillus amylovorus* Nakamura 1981 has also been provided.

Emended description of *Lactobacillus amylovorus* Nakamura 1981

Lactobacillus amylovorus (a.my.lo.vo'rus. Gr. neut. n. *amylon* starch; L. v. *vorare* to devour; N. L. masc. adj. *amylovorus* starch-devouring).

The following characteristics emended those reported in the original description by Nakamura [8] and in a list of species properties by Zheng *et al*. [3].

Cells are Gram-stain-positive, nonmotile, non-spore-forming, catalase-negative, oxidase-negative, facultatively anaerobic, homofermentative, and rod-shaped. The cells grow individually and in short chains. The agar colonies are white, convex, smooth, circular, complete, and opaque. The broth is turbid or clears after a few days owing to the settling of the cells. They produce both D- and L-lactate and small amounts of acetate but no gas from glucose or gluconate. Growth temperatures are as follows: optimum, 37 to 45 °C; minimum, 20 to 25 °C; and maximum, 45 to 50 °C. In the API 50 CH test system (incubation at 37 °C for 2 d), acids are produced from D-glucose, D-fructose, *N*-acetyl-glucosamine, esculin ferric citrate, and D-maltose, but not from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl-α-D-mannopyranoside, inulin, D-melezitose, xlitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate, and 5-keto-gluconate. Variable reactions for acid production from D-galactose, D-mannose, D-mannitol, methyl-α-D-glucopyranoside, amygdalin, arbutin, salicin, D-cellobiose, D-lactose, D-melibiose, D-sucrose, D-trehalose, D-raffionose, starch, glycogen, gentiobiose, and D-turanose are observed. In the API ZYM test system, positive reactions are observed for esterase (C4), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, and β-glucosidase. Negative reactions are obtained from alkaline phosphatase, lipase (C14), trypsin, α-chymotrypsin, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Various reactions are obtained from esterase lipase (C8), cystine arylamidase, and α-galactosidase. An extracellular amylolytic enzyme is then formed. Nitrate is not reduced to nitrite. Nicotinic acid, pantothenic acid, folic acid, and riboflavin are essential for growth, whereas thiamine is not required. $C_{14:0}$, $C_{16:0}$, $C_{18:1}$ ω9*c*, and C_{19} cyclo9, 10 are the major fatty acids in strain DSM 20531^T. The genome size of the type strain is 2.02 Mbp, and the mol% G+C content of the DNA is 37.8 (whole-genome analysis). This microorganism is a characteristic representative of the swine intestinal microbiota [50, 51], and has been isolated from other environments, such as sourdough, cattle waste-corn fermentation, spent mushroom substrates, and bovine feces. The type strain is ATCC 33620^T = CCUG 27201^T =CIP 102989^T = DSM 20531^T = JCM 1126^T = LMG 9496^T = NCAIM B.01458^T = NRRL B-4540^T, isolated from cattle waste-corn fermentation. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DSM 20531^T is AY944408. The genome sequence accession number for strain DSM 20531^T is AZCM00000000.

Description of *Lactobacillus amylovorus* subsp. *amylovorus* subsp. nov.

Lactobacillus amylovorus subsp. *amylovorus* (a.my.lo.vo'rus. Gr. neut. n. *amylon* starch; L. v. *vorare* to devour; N. L. masc. adj. *amylovorus* starch-devouring).

The description is essentially in agreement with that given above for the species *Lactobacillus amylovorus*, with the following modifications.

In the API 50 CH test system (incubation at 37 °C for 2 d), acids are produced from D-galactose, D-glucose, D-fructose, D-mannose, *N*-acetyl-glucosamine, esculin ferric citrate, salicin, D-maltose, D-trehalose, and gentiobiose, but not from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl-α-D-mannopyranoside, inulin, D-melezitose, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate, and 5-keto-gluconate. Variable reactions are observed for acid production from D-mannitol, methyl-α-D-glucopyranoside, amygdalin, arbutin, D-cellobiose, D-lactose, D-melibiose, D-sucrose, D-raffinose, starch, glycogen, and D-turanose. In the API ZYM test system, positive reactions are observed for esterase (C4), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, and β-glucosidase. Negative reactions are obtained from alkaline phosphatase, esterase lipase (C8), lipase (C14), trypsin, α-chymotrypsin, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. The reactions of the following enzymes vary: cystine arylamidase, α-galactosidase, and β-galactosidase. The type strain is ATCC 33620^T = CCUG 27201^T =CIP 102989^T = DSM 20531^T = JCM 1126^T = LMG 9496^T = NCAIM B.01458^T = NRRL B-4540^T, isolated from cattle waste-corn fermentation. At least three additional strains (YK3, YK6, and YK10) are included in this subspecies.

Description of *Lactobacillus amylovorus* subsp. *animalis* subsp. nov.

Lactobacillus amylovorus subsp. *animalis* (a.ni.ma'lis. L. gen. neut. n. *animalis*, of a living being, an animal).

Cells are Gram-stain-positive, nonmotile, non-spore-forming, catalase-negative, cytochrome oxidase-negative, facultatively anaerobic, homofermentative, and rod-shaped. The cells of BF125^T are 0.5–0.7 μm in width and 3.0–7.0 μm in length. Colonies grown on MRS agar plates at 37 °C for 3 d under anaerobic conditions are 1.0-2.0 mm in diameter and milky white, round, and glossy in appearance. This strain does not produce any gas from glucose; both D- and L-lactate are produced as end products of glucose (D/L, 40:60). Growth occurs at a temperature of 30–45 °C (optimum 37 °C), pH of 5.0–8.0 (optimum pH 6.0), and NaCl concentration of 1.0–3.0% (w/v). In the API 50 CH test system (incubation at 37 °C for 2 d), acids are produced from D-galactose, D-fructose, *N*-acetyl-glucosamine, esculin ferric citrate, D-cellobiose, D-maltose, D-lactose, D-sucrose, and starch, but not from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, amygdalin, arbutin, salicin, D-melibiose, inulin, D-melezitose, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-

keto-gluconate, and 5-keto-gluconate. Variable reactions have been observed for acid production from D-galactose, D-mannose, D-trehalose, and D-raffinose. In the API ZYM test system, positive reactions are obtained from esterase (C4), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, and β-glucosidase. Negative reactions are obtained from alkaline phosphatase, lipase (C14), trypsin, α-chymotrypsin, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. The reactions of the following enzymes vary: esterase lipase (C8) and α-galactosidase. Dextran is not produced from sucrose, and ammonia is not produced from arginine. $C_{16:0}$, $C_{18:1}$ ω9*c*, $C_{19:0}$ cyclo ω8*c,* and summed feature 10 are the major fatty acids in strain BF125^T. The genome size of the type strain is 1.98 Mbp, and the mol% G+C content of the DNA is 37.8 (whole-genome analysis). The type strain BF125^T (= MAFF 212522^T = DSM 115528^T) is isolated from bovine feces. At least five additional strains [BF186, Bifido-178-WT-3C (= DSM 107288), S60, AF08-3, and DSM 16698] are included in this subspecies. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BF125^T is LC771959. The INSDC accession number for the draft genome sequence of strain BF125^T is BTFR01.

AUTHOR STATEMENTS

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Conflicts of interest

K. Y. and H. T. are employees of Nihon Shokuhin Kako Co., Ltd.. H. K., T. K., and M. T. received research grants from Nihon Shokuhin Kako Co., Ltd.. The authors declare no conflicts of interest.

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Ethical approval

All animal studies were conducted in accordance with the Animal Care and Use Guidelines of NARO.

Consent for publication

Not applicable.

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ABBREVIATIONS

ANI, average nucleotide identity; COG, cluster of orthologous group; dDDH, digital DNA-DNA hybridization; iTOL, interactive tree of life; MRS, de Man, Rogosa, and Sharpe; NJ, neighbor-joining; ML, maximum likelihood; MP, maximum-parsimony; MIDI, microbial identification system.

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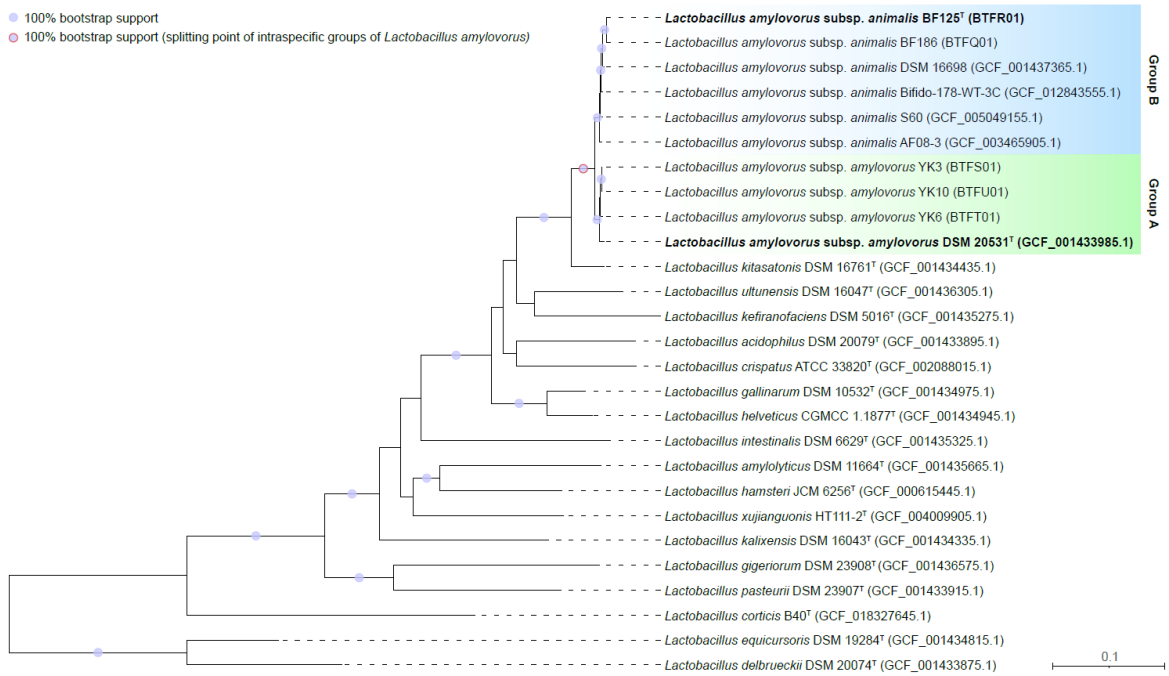

Fig. 1. Phylogenetic tree reconstructed via concatenated alignment of 166 core genes based on whole-genome sequences of various strains of *L. amylovorus* including the isolates BF125^T, BF186, YK3, YK6, and YK10, and the type strains of the closely related species of the genus *Lactobacillus*. The strains DSM 20531^T (in the intraspecific group A of *L. amylovorus*) and BF125^T (in group B) are in boldface. Bootstrap support of 100% is indicated on the nodes with solid circle filled by light blue (based on 1,000 replications). The tree is rooted using midpoint rooting. Bar, 0.1 substitutions per nucleotide position.

			Group A				Group B							
	INSDC accession number	strain	1	2	3	4	5	6	7	8	9	10	11	12
1	GCF 001433985.1	*L. amylovorus* subsp. *amylovorus* DSM 20531^T		97.70	97.93	97.91	96.21	96.28	96.31	96.30	96.30	96.50	91.42	79.58
2	BTFT01	*L. amylovorus* subsp. *amylovorus* YK6	85.2		98.65	98.51	96.40	96.46	96.23	96.37	96.07	96.48	91.15	79.25
3	BTFS01	*L. amylovorus* subsp. *amylovorus* YK3	87.3	92.1		99.56	96.43	96.43	96.3	96.19	96.35	96.42	91.32	79.37
4	BTFU01	*L. amylovorus* subsp. *amylovorus* YK10	87.8	92.6	98.1		96.30	96.32	96.32	96.20	96.30	96.44	91.08	79.27
5	BTFR01	*L. amylovorus* subsp. *animalis* BF125^T	74.1	75.2	74.1	73.8		99.96	97.92	98.03	97.88	97.71	90.83	79.85
6	BTFQ01	*L. amylovorus* subsp. *animalis* BF186	74.2	75.3	74.3	74.0	100.0		97.9	98.01	97.84	97.58	90.77	79.85
7	GCF 012843555.1	*L. amylovorus* subsp. *animalis* Bifido-178-WT-3C	75.6	74.8	74.8	74.7	87.1	87.2		97.96	98.61	98.1	91.07	79.88
8	GCF 005049155.1	*L. amylovorus* subsp. *animalis* S60	75.0	74.1	74.0	74.2	87.5	87.7	88.1		97.76	97.73	90.93	79.55
9	GCF 001437365.1	*L. amylovorus* subsp. *animalis* DSM 16698	75.2	74.0	75.0	75.0	85.4	85.5	90.9	86.6		97.80	90.86	79.70
10	GCF 003465905.1	*L. amylovorus* subsp. *animalis* AF08-3	77.1	75.1	75.8	76.1	85.5	85.6	86.0	86.8	87.0		90.63	79.24
11	GCF 001434435.1	*L. kitasatonis* DSM 16761^T	47.0	45.8	46.2	46.2	45.1	45.1	46.1	45.8	45.5	45.8		79.59
12	GCF 002088015.1	*L. crispatus* ATCC 33820^T	23.9	23.5	23.5	23.5	24.1	24.0	24.1	23.8	23.9	23.8	23.8	

ANI

dDDH

Fig. 2. Pairwise comparison of ANI (above the diagonal) and dDDH (below the diagonal) percentages among *L. amylovorus* and its closely related taxa.

559 **Table 1.** Differential phenotypic features and isolation sources of tested strains belonging to *L.*
560 *amylovorus* subsp. *amylovorus* or *L. amylovorus* subsp. a*nimalis.*

561 Strains in group A (*L. amylovorus* subsp. *amylovorus*): 1, DSM 20531^T; 2, YK3; 3, YK6; 4, YK10.
562 Strains in group B (*L. amylovorus* subsp. *animalis*): 5, BF125^T; 6, BF186; 7, DSM 16698; and 8, DSM
563 107288 (= Bifido-178-WT-3C). All tested characteristics were investigated under identical conditions
564 in the present study. Gray shading, positive; white shading, negative.

565

	Group A				**Group B**			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Isolation source:	cattle waste-corn fermentation	spent mushroom substrates	spent mushroom substrates	spent mushroom substrates	bovine feces	bovine feces	intestine of weaning piglets	porcine feces
Acid production from:								
D-Galactose	■	■	■	■	■		■	
D-Mannose	■	■	■	■		■		■
D-Mannitol		■	■	■				
Methyl-α-D-glucopyranoside	■		■					
Salicin	■	■	■	■				
D-Lactose					■	■	■	■
D-Melibiose		■	■					
D-Raffinose		■	■	■			■	■
Glycogen	■	■		■				
Gentiobiose	■	■	■	■				
D-Turanose	■		■					
Enzyme activity:								
Esterase lipase (C8)					■	■		
Cystine arylamidase			■		■	■	■	■
α-Galactosidase		■	■	■			■	■
β-Galactosidase		■	■		■	■	■	■

566