Lactococcus garvieae subsp. bovis subsp. nov., lactic acid bacteria isolated from wild gaur (Bos gaurus) dung, and description of Lactococcus garvieae subsp. garvieae subsp. nov.

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A taxonomic investigation was performed on a Gram-stain-positive coccus, designated strain BSN307\(^T\), isolated from gaur (Indian bison, Bos gaurus) dung based on phenotypic and molecular approaches. Based on the biochemical tests, cellular morphology and 16S rRNA gene sequence similarity, this strain was found to be a member of the genus Lactococcus and closely related to Lactococcus garvieae ATCC 49156\(^T\) (99.6% 16S rRNA gene sequence similarity) and Lactococcus formosensis 516\(^T\) (99.0%). However, DNA–DNA hybridization studies showed that the level of relatedness between strain BSN307\(^T\) and L. garvieae ATCC 49156\(^T\) was 75.8%, suggesting that it represented a novel subspecies of L. garvieae. The inability to grow in brain heart infusion (BHI) medium at pH 9.6, in tryptic soy agar (TSA) with 4% (w/v) NaCl and at 42 °C (MRS agar) clearly differentiated BSN307\(^T\) from L. garvieae ATCC 49156\(^T\). Rep-PCR fingerprint patterns, substantial differences in summed feature 8 (C\(_{18:1}\)ω7c/C\(_{18:1}\)ω6c), C\(_{19:0}\) cyclo ω8c and C\(_{16:0}\) also differentiated strain BSN307\(^T\) from the reference strain of L. garvieae. Moreover, analysis of the housekeeping genes pheS and rpoA revealed sequence similarities that were at the limit for species differentiation (92.2 and 97.8%, respectively). Combined genotypic and phenotypic data indicate that strain BSN307\(^T\) represents a subspecies of L. garvieae for which the name Lactococcus garvieae subsp. bovis subsp. nov. is proposed. The type strain is BSN307\(^T\) (=DSM 100577\(^T\) =MCC 2824\(^T\) =KCTC 21083\(^T\)).

Lactic acid bacteria (LAB) are found in many habitats such as dairy products, milk, fruits, vegetables and decaying organic matter. They are free living or found in association with animals as normal flora of the oral cavity, intestinal tract and vagina. Lactococcus was established as a separate genus distinct from the genus Streptococcus by Schleifer et al. (1985). The genus Lactococcus contains, at the time of writing, 11 recognized species: Lactococcus lactis, L. garvieae, L. piscium, L. plantarum, L. raffinolactis, L. fujensis, L. chungangensis, L. taiwanensis, L. formosensis, L. hircilactis and L. laudensis (Cai et al., 2011; Chen et al., 2014; Meucci et al., 2015). In the present study, a LAB strain, designated as BSN307\(^T\), isolated from the dung of wild gaur (Indian bison, Bos gaurus) was studied based on 16S rRNA and housekeeping gene sequence analyses, phenotypic characteristics, rep-PCR, fatty acid methyl ester analysis and DNA–DNA hybridization techniques to establish its taxonomic position. Phylogenetic analysis revealed that this novel strain is closely related to L. garvieae. This probiotic culture is noted for the production of a phenolic compound with antioxidant and anticancer properties (Varsha et al., 2014, 2015) in addition to the production of a novel bioactive peptide (our unpublished data). Based on the presented information, strain BSN307\(^T\) is considered to represent a novel subspecies of L. garvieae.

Strain BSN307\(^T\), noted for its antifungal activity, was isolated from a gaur dung sample, collected from Silent Valley National Park, which is a part of the Western Ghats' natural world heritage site in India. The sample was inoculated in de Man Rogosa Sharpe medium (MRS; Himedia) and bacterial isolates were obtained by serial dilution on MRS agar (Himedia) plates containing 1% CaCO\(_3\) after 24h of incubation at 30 °C. L. garvieae CCUG 32208\(^T\) was obtained from Culture Collection, University of...
Gothenburg (CCUG). The two strains were preserved in 20 % (v/v) glycerol at –70 °C.

The eight isolated strains of LAB from gaur dung were designated as BSN301–BSN308, and checked for cell morphology by light microscopy (Leica), Gram staining, catalase, NaCl tolerance and growth pattern in MRS broth. Of the eight strains, BSN307\(^{T}\) was selected for further studies. Chromosomal DNA of BSN307\(^{T}\) was extracted and amplification of the 16S rRNA gene was performed with primers 27F and 1492R (Fernandez et al., 2010). Sequencing of the 16S rRNA gene was performed using an ABI 3500 (Applied Biosystems) genetic analyser according to the instructions of the manufacturer. Sequence similarity searches were performed using the EzTaxon-e server (Kim et al., 2012) and sequences for phylogenetic analysis were obtained from the same source. All sequences were aligned by using MUSCLE software (Edgar, 2004). Distances were calculated according to Kimura’s two-parameter model (Kimura, 1980). Phylogenetic trees were reconstructed using the neighbour-joining method (Saitou & Nei, 1987), with bootstrap analysis based on 1000 replications. The program MEGA version 6.0 (Tamura et al., 2013) was used for all the analyses.

Cell morphology and the presence of spores were examined by microscopy. Growth at pH 9.6 in brain heart infusion (BHI; Merck) medium, growth in the presence of 4 % (w/v) NaCl in tryptic soy medium (Himedia) and growth at 42 and 45 °C in MRS agar were assessed (Eldar et al., 1999). Growth in nutrient agar with 0.5 % (w/v) glucose, 0.5 % (w/v) yeast extract and 6.5 % (w/v) NaCl, and arginine dihydrolase activity in arginine dihydrolase media (Himedia) were checked at 30 °C. Acid production from carbohydrates was determined with API 50 CHL strips (bioMérieux) and haemolytic activity was checked on 5 % sheep blood agar (Himedia) after incubation at 30 °C for 48 h. Major metabolic end products were detected by HPLC (Shimadzu) using a Phenomenex relex ROA column with detection at 254 nm. The enzymatic properties of strain BSN307\(^{T}\) and \(L.\) garvieae CCUG 32208\(^{T}\) were determined using Himedia Hi25 kits according to the instructions of the manufacturer.

rep-PCR patterns, using primers (GTG)\(_3\), ERIC (ERIC1 and ERIC2) and SERE, which were previously demonstrated to be effective in characterization of \(Lactococcus\) species and subspecies, were analysed as described previously (Cho et al., 2008; Pérez et al., 2010). For DNA–DNA hybridization experiments, genomic DNA was extracted and purified with additional RNase treatment. DNA suspended in 2x SSC was used for the analysis. The analysis was performed by fluorimetry using a Step One Plus Real-Time PCR system (Applied Biosystems) fitted with a 96-well thermal cycling block in 96-well plates as described and evaluated before (Gillis et al., 1970; Ley et al., 1970; Loveland-Curtze et al., 2011). The DNA G+C content was determined based on the melting temperature (\(T_m\)) according to Gonzalez & Saiz Jimenez (2002). Amplification and sequencing of the housekeeping genes pheS and rpoA and cellular fatty acid analysis were carried out as described before (Chen et al., 2014). For cellular fatty acid analysis, 48-h-old cells grown in MRS broth at 30 °C were used, and analysis of fatty acid methyl esters was performed by using the Sherlock Microbial Identification System (version 6.1), according to the instructions of the Microbial Identification System (MIDI). Results of analyses were calculated from the mean of triplicate determinations.

The 16S rRNA gene sequence (1404 nt) was obtained for strain BSN307\(^{T}\). The BLAST results of the 16S rRNA gene sequence of this strain (GenBank accession number KM261818) indicated 99.6 % similarity to \(L.\) garvieae ATCC 49156\(^{T}\) and 99.0 % similarity to \(L.\) formosensis 516\(^{T}\). Other close relatives were the type strains of \(L.\) lactis subspecies (92.7–93.4 %), \(L.\) taiwanensis (93.0 %) and \(L.\) fujiensis (92.4 %). Phylogenetic analysis based on 16S rRNA gene sequences revealed that the novel strain BSN307\(^{T}\) belongs to the species \(L.\) garvieae (Fig. 1).

Colonies of strains BSN301–BSN308 were cream-coloured, opaque, round and convex on MRS plates. Cells were catalase-negative and microscopic analysis revealed Gram-stain-positive, coccoid or ovoid-shaped cells present singly or in short chains. No spores and mycelium were observed and cells could not grow on TSA with 4 % (w/v) NaCl. Strain BSN307\(^{T}\) was selected for further studies. Unlike \(L.\) garvieae CCUG 32208\(^{T}\), strain BSN307\(^{T}\) failed to grow at 42 and 45 °C, on pH 9.6 BHI medium and on nutrient agar containing 0.5 % glucose, 0.5 % yeast extract and 6.5 % NaCl. These specific traits for the identification of \(L.\) garvieae (Eldar et al., 1999) clearly differentiated BSN307\(^{T}\) from the type strain and other strains of \(L.\) garvieae. Another difference was the delayed arginine dihydrolase reaction of the novel strain when compared to the reaction for the type strain of \(L.\) garvieae. Acid production from carbohydrates and enzyme profiles were similar for BSN307\(^{T}\) and \(L.\) garvieae CCUG 32208\(^{T}\) (Table 1). Both the strains were \(\alpha\) haemolytic on sheep blood agar and had l-lactic acid as a major metabolic end product.

Due to the high 16S rRNA gene sequence similarity between BSN307\(^{T}\) and \(L.\) garvieae CCUG 32208\(^{T}\), DNA–DNA hybridization (DDH) was performed. A value of 75.8 % was obtained between the two strains; according to Meier-Kolthoff et al. (2014) a DDH value of 79 % can be used to separate bacterial subspecies, and therefore strain BSN307\(^{T}\) represents a subspecies of \(L.\) garvieae. The DNA G+C content of strain BSN307\(^{T}\) was 38.1 mol%, which was similar to that for \(L.\) garvieae CCUG 32208\(^{T}\) (38.3–38.7 mol%). The rep-PCR pattern analyses showed clear differences between BSN307\(^{T}\) and \(L.\) garvieae CCUG 32208\(^{T}\) with all the primers checked in both the number of bands and the molecular weight of bands present (Fig. S1, available in the online Supplementary Material). The rep-PCR pattern analysis previously used for characterization of \(L.\) lactis subspp. \(lactis\) L105\(^{T}\) demonstrated that the pattern is similar for strains belonging to the same subspecies but different among subspecies (Pérez et al., 2010), which
fatal confirms the subspecies status of BSN307\textsuperscript{T}. Analysis of the housekeeping genes \textit{pheS} (GenBank accession number KU319451) and \textit{rpoA} (GenBank accession number KU319452) of strain BSN307\textsuperscript{T} revealed high levels of similarity (99.7 and 99.6\%, respectively) to those of \textit{L. formosensis} 516\textsuperscript{T}. However, \textit{pheS} and \textit{rpoA} gene sequence similarities between BSN307\textsuperscript{T} and \textit{L. garvieae} CCUG 32208\textsuperscript{T} were at the limit for species differentiation (Naser \textit{et al.}, 2007) (92.2 and 97.8\%, respectively), which further separated BSN307\textsuperscript{T} from \textit{L. garvieae} CCUG 32208\textsuperscript{T} (Figs S2 and S3). Differences in the composition and amount of cellular fatty acids were found. The major fatty acids of strain BSN307\textsuperscript{T} were C\textsubscript{16:0} (34.9\%) and summed feature 8 (C\textsubscript{18:1ω7c/C18:1ω6c} 34.5\%) and for \textit{L. garvieae} CCUG 32208\textsuperscript{T} were C\textsubscript{16:0} (44.0\%) and C\textsubscript{19:0} cyclo ω8c (28.8\%). Substantial differences in summed feature 8, C\textsubscript{19:0} cyclo ω8c and C\textsubscript{16:0} differentiated strain BSN307\textsuperscript{T} from \textit{L. garvieae} CCUG 32208\textsuperscript{T}. Complete fatty acid profiles are given in Table S1.

In conclusion, this study shows that strain BSN307\textsuperscript{T}, isolated from wild gaur dung, has phenotypic and genotypic differences from \textit{L. garvieae} CCUG 32208\textsuperscript{T} despite sharing high 16S rRNA gene sequence similarity. On the evidence presented, we consider that strain BSN307\textsuperscript{T} merits separate subspecies status, and for which the name \textit{L. garvieae} subsp. \textit{bovis} nov. is proposed. Tests which serve to differentiate strain BSN307\textsuperscript{T} from \textit{L. garvieae} CCUG 32208\textsuperscript{T} are given in Table 1. According to Rule 46 of the Bacteriological Code (Lapage \textit{et al.}, 1992), the description of a novel subspecies which excludes the type of the species automatically creates the subspecies \textit{L. garvieae} subsp. \textit{garvieae} for which the type strain is ATCC 49156\textsuperscript{T}.

**Description of \textit{Lactococcus garvieae} subsp. \textit{bovis} subsp. nov.**

\textit{Lactococcus garvieae} subsp. \textit{garvieae} subsp. nov. (\textit{garvi}.ae. N.L. gen. n. \textit{garvieae} of Garvie, named for E. I. Garvie, a British microbiologist).

Possesses the characteristics previously described by Collins \textit{et al.} (1983) for the species \textit{Lactococcus garvieae}. Colonies are cream-coloured, opaque, round and convex on MRS plates. Cells are Gram-stain-positive, catalase-negative, non-spore-forming, non-mycelium-forming, coccoid or ovoid-shaped and are present singly or in short chains. Growth occurs on BHI medium at pH 9.6, on TSA with 4\% (w/v) NaCl and at 42°C. Major fatty acids are C\textsubscript{16:0} and C\textsubscript{19:0} cyclo ω8c. Given its phylogenetic placement based on 16S rRNA gene sequence analysis, \textit{L. garvieae} subsp. \textit{garvieae} belongs to the phylogenetic subgroup with \textit{L. formosensis} as the closest related member. Differentiation between these two is possible based on analysis of the 16S rRNA gene, housekeeping genes \textit{rpoA} and \textit{pheS}, and by DNA–DNA hybridization.

The type strain is ATCC 49156\textsuperscript{T} (=CCUG 32208\textsuperscript{T}=JCM 10343\textsuperscript{T}).

**Description of \textit{Lactococcus garvieae} subsp. \textit{bovis} subsp. nov.**

\textit{Lactococcus garvieae} subsp. \textit{bovis} subsp. nov. (bo’vis. L. gen. n. \textit{bovis} of Indian bison, of a bovine, referring to the isolation source).
**Data from the study of Eldar**

The type strain is BSN307

Data were obtained from:

+, Positive; –, negative; NA, not available.

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<th>Characteristic</th>
<th>1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>3&lt;sup&gt;b&lt;/sup&gt;</th>
<th>4&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>Growth with 4 % (w/v) NaCl</td>
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<td>+</td>
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<td>+*</td>
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<td>−</td>
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Data were obtained from: <sup>a</sup> this study; <sup>b</sup> Chen et al. (2014); <sup>c</sup> Pérez et al. (2010).

<sup>†</sup>Summed feature 8 comprised C<sub>19:1</sub>ω7c/C<sub>18:1</sub>ω6c.

**Morphology and carbohydrate utilization pattern** are as given for L. *garvieae* subsp. *garvieae* subsp. nov. Colonies are α haemolytic and produce l-lactic acid as the major metabolic end product. However, considerable phenotypic differences are noted. Cannot grow on BHI medium at pH 9.6, or on TSA with 4 % (w/v) NaCl and growth does not occur at 42 °C. The DNA G+C content is 38.1 mol%. Major cellular fatty acids are C<sub>16:0</sub> and summed feature 8 (C<sub>18:1</sub>ω7c/C<sub>18:1</sub>ω6c). Substantial differences in summed feature 8, C<sub>19:0</sub> cyclo ω8c and C<sub>16:0</sub> differentiate the type strain from *L. garvieae* CCUG 32208<sup>T</sup>. Genotypic differences as observed from DDH experiments, housekeeping gene sequence analysis and DNA fingerprinting patterns, based on rep-PCR pattern analysis, clearly differentiate it from *L. garvieae* subsp. *garvieae* subsp. nov.

The type strain is BSN307<sup>T</sup> (=DSM 100577<sup>T</sup>=MCC 2824<sup>T</sup>=KCTC 21083<sup>T</sup>).

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**References**


