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Enterococcus montenegrensis sp. nov., isolated from artisanal Montenegrin dry sausage

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Abstract

A novel, Gram-positive, facultative anaerobe, coccoid and non-motile bacterium, designated as CoE-012-22^T was isolated from dried beef sausage (the original name in Montenegro is Govedji Kulen) manufactured in the municipality of Rozaje (Montenegro) in 2021. Cells of this strain were oxidase- and catalase-negative. Growth occurred at 4–50°C, at pH 5.0–8.0 and with 0–6.5% (w/v) NaCl in diverse growth media. MALDI-TOF analysis identified the strain as *Enterococcus canintestini* (log score 2). Phylogenetic analysis of the 16S rRNA gene and whole genome sequences assigned the strain to the genus *Enterococcus*. The closest relatives were *E. canintestini* DSM 21207^T and *E. dispar* ATCC 51266^T with 16S rRNA gene sequence pairwise similarities of 99.34 and 98.59%, respectively. The average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) values between isolate CoE-012-22^T and other enterococci species were below the thresholds for species delineation thresholds (95.0% ANI; 70.0% dDDH) with maximum identities of 84.13% (ANIb), 86.43% (ANIm) and 28.4% (dDDH) to *E. saigonensis* JCM 31193^T and 70.97% (ANIb), 88.99% (ANIm) and 32.4% (dDDH) to *E. malodoratus* ATCC 43197^T. Two unknown *Enterococcus* isolates, *Enterococcus* sp. MJM12 and *Enterococcus* SMC-9, showed identities of 99.87 and 99.94% (16S rRNA), 98.57 and 98.65% (ANIb), 98.93 and 99.02% (ANIm), and 89.8 and 90.0% (dDDH) to strain CoE-012-22^T and can therefore be regarded as the same species. Based on the characterization results, strain CoE-012-22^T was considered to represent a novel species, for which the name *Enterococcus montenegrensis* sp. nov. is proposed. The type strain is CoE-012-22^T (=DSM 115843^T=NCIMB 15468^T).

INTRODUCTION

Enterococci are Gram positive, facultative anaerobe micro-organisms belonging to the group of lactic acid bacteria. The genus *Enterococcus* comprises currently 83 species of which 62 are validly published (https://lpsn.dsmz.de/search?word=Enterococcus). The type species of the genus is *Enterococcus faecalis*. Enterococci are widespread in the environment, are members of the intestinal commensal flora of humans and animals, have probiotic properties [1] conferring beneficial health effects [2–4], and have been reported as opportunistic pathogens [2, 3]. Enterococci also play an essential role in the fermentation processes of a variety of

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Abbreviations: ANI, average nucleotide identity; ARG, antimicrobial resistance gene; dDDH, digital DNA–DNA hybridization; MGE, mobile genetic element; PGAP, Prokaryotic Genome Annotation Pipeline; rMLST, ribosomal multilocus sequence typing; TYGS, Type Strain Genome Server; VG, virulence gene; WGS, whole genome sequencing.

The *Enterococcus montenegrensis* sp. nov. CoE-012-22^T whole genome shotgun project has been deposited in DDBJ/ENA/GenBank under accession no PRJNA940651. The version described in this paper is the first version, CP120467. The raw sequence reads have been deposited in the Sequence Read Archive under accession nos. SRR23759723 and SRR23693802. The 16S rRNA gene sequence has been deposited under accession no. OQ627393.

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Four supplementary figures and four supplementary tables are available with the online version of this article.

food products. In artisanal cheese and meat products, enterococci are considered indispensable for the specific aroma, texture, flavour and taste [5–9]. Endemic strains obtained from artisanal food products might be a valuable resource for future food production [10–12]. Enterococci also enhance the biological safety of food products by preventing bacterial foodborne diseases through the production of a variety of antimicrobial substances such as bacteriocins, organic acids and hydrogen peroxide that inhibit the growth of diverse foodborne pathogens and spoilage microorganisms [13].

However, despite the proven positive health effects, the importance and the long and safe application of enterococci [14], their use in the food production is controversial due to the emergence of multidrug-resistant strains [15, 16]. Consequently, the genus *Enterococcus* has not obtained the "generally recognized as safe" [17] and the qualified presumption of safety statuses defined by the European Food Safety Authority based on the absence of resistance and virulence markers [18]. Therefore, safety assessment for enterococci used in food is based on a case-by-case investigation [19], analysing the resistance and virulence potential of the respective strains [20].

In a study focused on the characterization of the bacterial population of traditional dried Montenegrin sausages, with the aim to explore their diversity and provide genetic information as a basis for the selection of strains for future manufacturing of artisanal food products [12], we obtained a bacterial isolate unassignable to a known species. The novel strain belongs to the genus *Enterococcus* and the proposed name for this strain is *Enterococcus montenegrensis* sp. nov.

ISOLATION AND MAINTENANCE

Strain CoE-012-22^T was isolated from beef dried sausage 'Govedji Kulen' manufactured in the municipality of Rozaje (Montenegro) in 2021. Samples were homogenized in buffered peptone water using a stomacher according to the ISO 15214:1998 method [21]. The strain was isolated from a diluted cell suspension plated on Columbia agar +5% sheep blood (COS) (bioMérieux) and incubated under aerobic conditions at 37°C for 24 h. A white colony was purified and maintained on COS and stored in glycerol stocks at −80°C. The type strain CoE-012-22^T was deposited at the German Collection of Microorganisms and Cell Cultures GmbH (DSM 115843^T) and the National Collection of Industrial Food and Marine Bacteria (NCIMB 15468^T).

Reference strains used for comparison with strain CoE-012-22^T were purchased from the German Collection of Microorganisms and Cell Cultures GmbH (*E. canintestini* DSM 21207^T, *E. devriesei* DSM 22802^T, *E. dispar* DSM 6630^T=ATCC 51266^T), the American Type Culture Collection (*E. faecalis* ATCC 29212) and the Culture Collection University of Goethenburg (*E. saigonensis* CCUG 68827^T=JCM 31193^T).

16S rRNA GENE PHYLOGENY

The 16S RNA gene sequence was extracted from the original genome file with Barnapp GL3.0 using the DFAST tool version 1.6.0. annotation server [22]. Basic Local Alignment Search Tool (BLAST) [23] analysis revealed 98.59% similarity of the 16S rRNA genes between CoE-012-22^T and *E. dispar* ATCC 51266^T (99% query coverage) and 99.34% similarity between CoE-012-22^T and *E. canintestini* DSM 21207^T (97% query coverage). CoE-012-22^T showed similarity values of the 16S rRNA with two unknown enterococci strains, MJM12 (JAFLVT000000000) [24] and SMC-9 (OL689132), of 99.87 and 99.94% respectively. Strain CoE-012-22^T differed from *Enterococcus* sp. MJM12 by A201T transversion and a C596T transition and from *Enterococcus* sp. SMC-9 by a C596T transition. Positions were numbered according to the *Escherichia coli* 16S rRNA gene reference (J01859.1) [25].

A comparison of the 16S rRNA gene of CoE-012-22^T with the closest related enterococci 16S rRNA genes and the two unknown enterococci strains MJM12 and SMC-9 was carried out using MEGA version 11.0.13 [26] Multiple sequence alignments were performed with the Clustal_W program implemented in the MEGA software package. Phylogenetic trees were reconstructed based on aligned sequences using the neighbour-joining (NJ) [27] algorithm. The Jukes–Cantor model [28] was used to calculate the genetic distances for the NJ analysis. Bootstraps resampling with 1000 replications was employed to evaluate the confidence values of nodes in the phylogenetic tree [29]. Treatment of gaps/missing data was performed with pairwise deletion for the reconstruction of the phylogenetic tree. *Escherichia coli* DSM 300383^T was used as the outgroup. Strain CoE-012-22^T clustered together with *Enterococcus* sp. MJM12 and *Enterococcus* sp. SMC-9. The three strains formed a small branch and separated from other species in the NJ tree (Fig. 1).

GENOMIC ANALYSIS

High molecular weight DNA was isolated from overnight cultures grown on COS agar using the MagAttract HMW DNA Kit (Qiagen) following the manufacturer's instruction for Gram-positive bacteria. The genomic library was prepared using the Illumina DNA Prep (M) kit (Illumina) and 2×150 pb paired-end sequencing was performed on a NextSeq2000 instrument (Illumina). Raw reads were *de novo* assembled using SPAdes (version 3.11.1) [30]. The obtained contigs were filtered for a minimum coverage of 5× and a minimum length of 200 base pairs using SeqSphere+ software version 8.2.0 (Ridom) [31]. The Rapid Barcoding Sequencing kit (SQK-RBK004, Oxford Nanopore Technologies) and a FLO-MIN106D R9.4.1 SpotON flow cell

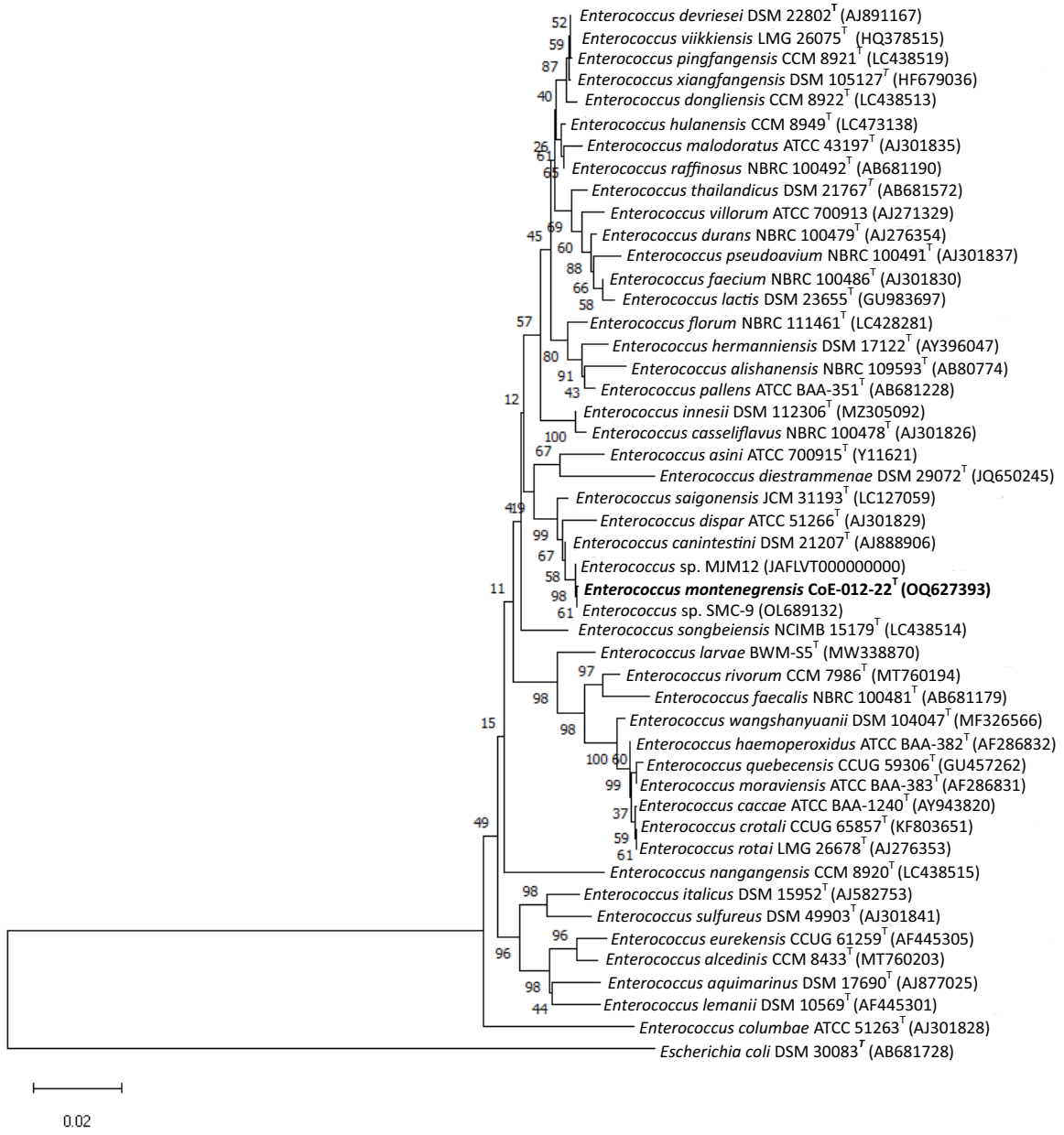

Fig. 1. 16S rRNA gene-based neighbour-joining tree showing the phylogenetic positions of *E. montenegrensis* CoE-012-22^T, *Enterococcus* sp. MJM12 and *Enterococcus* sp. SMC-9 and 44 *Enterococcus* sp. strains and type strains created with MEGA version 11.0.13. *Escherichia coli* DSM 30083^T was used as the outgroup. Numbers in brackets show GenBank accession number. Bootstraps values are shown at the branches. Bar, 0.02 substitutions per nucleotide position.

were used for long-read sequencing on a MinION Mk1C device, according to the manufacturer's instructions (Oxford Nanopore Technologies). A total of 45867 Nanopore reads were obtained using Guppy version 6.1.5 [32] in fast base-calling mode and filtered using Filtlong version 0.2.1 (https://github.com/rrwick/Filtlong) with the following parameters: min_length, 1000; keep_percent, 90; target_bases, 500000000. A total of 7483495 Illumina reads were quality controlled using FastQC version 0.11.9. Twelve read files were subsampled from the long read sequences and three assemblers were used to create four assemblies each (Flye version 2.9.1-b1780 [33], Miniasm version 0.3-r179 [34] and Raven version 1.8.1 [35]). For these 12 assemblies, Trycycler (version 0.5.4) [36] was used to create a consensus assembly and BWA (version 0.70.17) [37] and Polypolish (version 0.5.0) were used to polish the assembly with the Illumina reads. A complete genome was obtained with a mean coverage of 380-fold, a genome size of

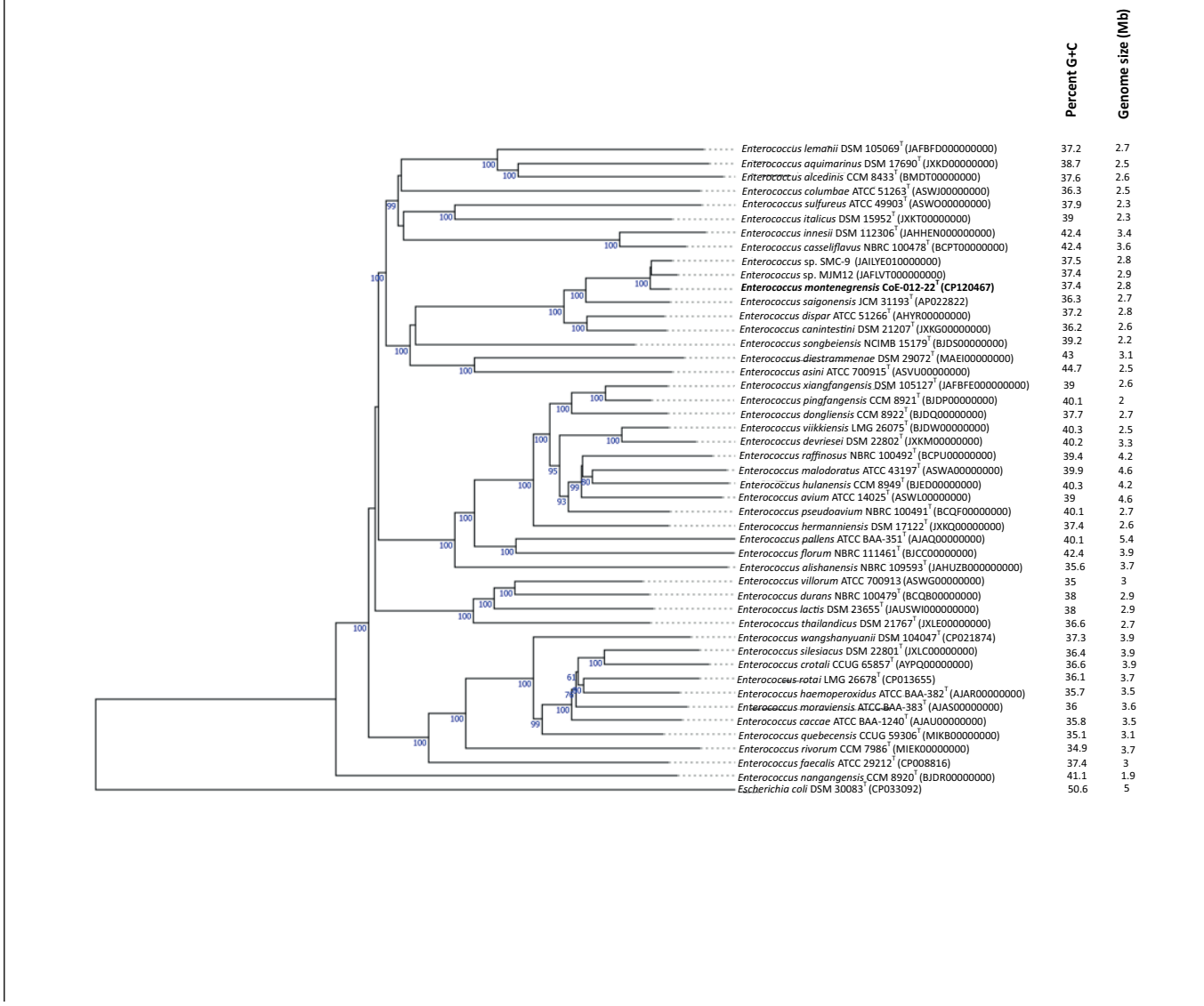

Fig. 2. Whole-genome-based tree from TYGS of *E. montenegrensis* CoE-012-22^T from Govedji Kulen sausage, *Enterococcus* sp. MJM12 and *Enterococcus* sp. SMC-9 and 44 *Enterococcus* sp. strains and type strains available in NCBI. *Escherichia coli* DSM 30083^T was used as the outgroup. The numbers on the tree nodes display confidence (100, maximum confidence). Numbers in brackets show GenBank accession numbers.

2.8 Mb and a DNA G+C content of 37.4mol%. The NCBI Prokaryotic Genome Annotation Pipeline [38] identified 2705 genes, 38 pseudogenes and 87 RNA genes (18 complete rRNA, 65 tRNA and 4 ncRNAs).

Genomic analysis using ribosomal multilocus sequence typing (rMLST) [39], digital DNA–DNA hybridization (dDDH) and whole genome sequence (WGS) analysis (Fig. 2) using the Type Strain Genome Server (TYGS) tool (https://tygs.dsmz.de, accessed 20 October 2023) [40] and average nucleotide identity (ANI) analysis using JSpecies version 3.9.7 [41] were performed. rMLST did not assign *E. montenegrensis* CoE-012-22^T to a known species. rMLST analysis identified 53 ribosomal genes for strain CoE-012-22^T matching 50 loci identical to undefined *Enterococcus* sp. and three loci identical to both *E. saigonensis* JCM 31193^T (GCA_011397115.1) and *E. dispar* ATCC 51266^T (GCA_000407585.1). dDDH using the formula d4 revealed 32.4% identity with *E. malodoratus* ATCC 43197^T, 31.3% with *E. devriesei* DSM 22802^T, 28.4% with *E. saigonensis* JCM 31193^T, 25.8% with *E. dispar* ATCC 51266^T and 24.8% with *E. canintestini* DSM 21207, and 89.8 and 90.9% similarity to *Enterococcus* sp. MJM12 and *Enterococcus* sp. SMC-9, respectively. ANIb (cut-off >95% identity) showed 81.20% identity with *E. canintestini* DSM 21207^T, 84.13% with *E. saigonensis* JCM 31193^T, 81.99% with *E. dispar* ATCC 51266^T, 71.10% with *E. devriesei* DSM 22802^T, 70.97% with *E. malodoratus* ATCC 43197^T and 71.59% with *E. faecalis* ATCC 29212, respectively.

A similarity of 98.57 and 98.65% (ANIb) was found between *Enterococcus* sp. MJM12 and *Enterococcus* sp. SMC-9 and *E. montenegrensis* CoE-012-22^T (Table 1; Fig. S1 available in the online version of this article). The number of coding sequences in strain CoE-012-22^T is 2580, while *Enterococcus* sp. MJM12 and *Enterococcus* sp. SMC-9 have 2720 and 2614, respectively.

Using the cgMLST Target Definer tool included in SeqSphere+ version 8.5.1 (Ridom) with default settings (90% gene identity and 100% gene overlap) [42] using strain CoE-012-22^T as the reference genome and *Enterococcus* sp. MJM12 and *Enterococcus* sp. SMC-9 as query genomes, a core genome comprising 2037 genes was obtained. *E. saigonensis* JCM 31193^T, *E. dispar* ATCC 51266^T, *E. canintestini* DSM 21207^T and *E. faecalis* ATCC 29212 shared only 250 (12.3%), 179 (8.8%), 139 (6.8%) and 6(0.3%) genes with this *E. montenegrensis* CoE-012-22^T core genome scheme. *E. montenegrensis* CoE-012-22^T differed from *Enterococcus* sp. MJM12 and *Enterococcus* sp. SMC-9 by 1880 and 1887 alleles in the defined core genome (Fig. S2). A core genome comprises a species-specific set of genes present in every strain of a species, which makes it a useful tool not only for strain typing but also for accurate species identification.

For detailed comparison of gene presence and absence, gene prediction was performed using Prodigal version 2.6.3 [43] with default settings for all strains to reduce the different gene-identification algorithm bias. Orthofinder version 2.5.4 was used to determine orthologous groups [44]. Furthermore, a species tree was generated using Orthofinder [45, 46]. The species tree was inferred with the Orthofinders default species tree method STAG [45]. Orthofinder analysis revealed that our strain expresses a unique pattern in comparison with other enterococci strains. The heatmap shows the difference in presence/absence of orthologous gene groups detected in the respective samples (Fig. 3). There are major and minor clusters of orthologous genes shared between the compared strains. Potential gene functions of orthologous groups were identified by comparing the NCBI-PGAP annotation with groups where differences between *E. montenegrensis* CoE-012-22^T and *Enterococcus* sp. MJM12 and/or *Enterococcus* sp. SMC-9 and/or other enterococci were observed. *E. montenegrensis* CoE-012-22^T has a total of 2395 orthologous groups shared with at least one of the compared strains. The intersection of the orthologous groups of *E. montenegrensis* CoE-012-22^T with related strains/species is as follows: *Enterococcus* sp. MJM12 (2299, ~96%), *Enterococcus* sp. SMC-9 (2272, ~95%), *E. saigonensis* JCM 31193^T (2079, ~87%), *E. dispar* ATCC 51266^T (2069, ~86%), *E. canintestini* DSM 21207^T (1998, ~83%), *E. faecalis* ATCC 29212(1644, ~69%) and *E. songbeiensis* 85-4^T (1558, ~65%) (Figs 3 and 4). Information about the presence or absence of the orthologous groups in the strains *E. montenegrensis* CoE-012-22^T, *Enterococcus* sp. MJM12, *Enterococcus* sp. SMC-9 and other enterococci are shown in the supplementary material (Table S1).

The Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg/genes.html) was used to describe the function and products of the genes present in CoE-012-22^T. *Enterococcus montenegrensis* CoE-012-22^T presented some specific genes which were absent in *Enterococcus* sp. MJM12 and *Enterococcus* sp. SMC-9 (Table 2). Briefly, specific genes in our strain which could provide beneficial properties to the food are related to hydrolase activity on ester bonds, carbohydrate transmembrane transporter activity and tagatose-bisphosphate aldolase activity. Other genes found are associated with sialic acid lyase activity, DNA-binding transcription factor activity, flavin mono nucleotide (FMN) binding and histidine-containing phosphotransfer (HPt). These activities are related to signalling, immunity and gene expression regulation (Table 2).

Hydrolase activity in bacteria cleaves proteins in meat to peptides and free amino acids, which contribute to the development of flavours and textures [47]. The carbohydrate transmembrane transporter activity is crucial in bacterial since it allows the use of carbohydrates as source of energy; therefore, it is important in nutrient acquisition and energy production, osmoregulation and cell communication, in addition to flavour development. Tagatose-bisphosphate aldolase activity is involved in the catabolism of tagatose, a rare ketohexose sugar, providing the bacteria with energy and carbon.

Safety assessment of strain *E. montenegrensis* CoE-012-22^T was performed using tools from Center for Genomic Epidemiology (www.genomicepidemiology.org; accessed 28 February 2023) using default parameters to detect antimicrobial resistance genes (ARGs) [48, 49], virulence genes (VGs) [50], plasmids [51], mobile genetic elements (MGEs) and pathogenic factors [52]. In addition, the Comprehensive Antibiotic Resistance Database (CARD) [53] was used to detect ARGs. Strain CoE-012-22^T carried no ARGs, VGs, MGEs nor plasmids. Pathogen-finder predicted it as non-pathogenic. CARD database (using perfect and complete genes criteria) revealed that strain CoE-012-22^T carried *VanY* and *VanT* (31.42 and 33.88% identity; Table S2). *Enterococcus* sp. MJM12 and *Enterococcus* sp. SMC-9 carried *tetM* and *VanT* in the VanG cluster (33.88% identity) and *VanY* in the VanB cluster (34.55% identity), the repUS1 plasmid, and ISLgar5 MGE (Table S2).

PHENOTYPIC CHARACTERISTICS

Transmission electron microscopy was used to determine bacterial cell morphology by negative staining. Bactericin treated carbon-coated pioloform copper grids were immersed in a droplet of freshly prepared bacterial suspension (4°C cold PBS, pH 6.8) for 20 min. After rinsing with double distilled water, the grids were immediately stained with 0.5% uranyl acetate. Grids were air-dried and analysed in a Zeiss 906 at 80 KV. Cells of CoE-012-22^T were coccoid, approximately 1.8 µm long and 0.7 µm wide and arranged in pairs or short chains (Fig. 5). Gram staining [54], oxygen requirement (AnaeroGen, ThermoFisher) and activity of catalase and oxidase (BD BBL oxydase, BD), and H_2S production (triple sugar iron agar) were determined. *E. montenegrensis*

Table 1. Overview on the number of proteins, genome length (Mbp), DNA G+C content (mol%), dDDH (%) (formula d4; cut-off >70%), ANIb (cut-off >95% identity) and ANIm (cut-off >95% identity) of the strain Enterococcus montenegrensis CoE-012-22^T compared to other enterococci type-strains

Strains: 1, *E. montenegrensis* CoE-012-22^T; 2, *E. canintestini* DSM 21207^T; 3, *E. dispar* ATCC 51266^T; 4, *E. devriesei* DSM 22802^T; 5, *E. malodoratus* ATCC 43197^T; 6, *E. saigonensis* JCM 31193^T; 7, *E. faecalis* ATCC 29212; 8, *Enterococcus* sp. MJM12; 9, *Enterococcus* sp. SMC-9.

Characteristics	1	2	3	4	5	6	7	8	9
Accession number	GCF_029983095.1	JXKG01	ASWK01	JXKM01	AJAK01	NZ_APO22822.1	NZ_CP008816.1	GCF_017316025.1	GCF_021011285.1
No. of proteins	2617	2517	2637	3135	4499	2700	2907	2767	2646
Genome length (Mbp)	2.8	2.6	2.8	3.3	4.6	2.8	3.0	2.9	2.8
DNA G+C content (mol%)	37.4	36.2	37.2	40.2	39.9	36.3	37.4	37.4	37.5
dDDH (%)	–	24.8	25.8	31.3	32.4	28.4	26	89.8	90.9
ANIb	–	81.20	81.99	71.10	70.97	84.13	71.59	98.57	98.65
ANIm	–	85.02	85.76	89.22	88.99	86.43	85.78	98.93	99.02

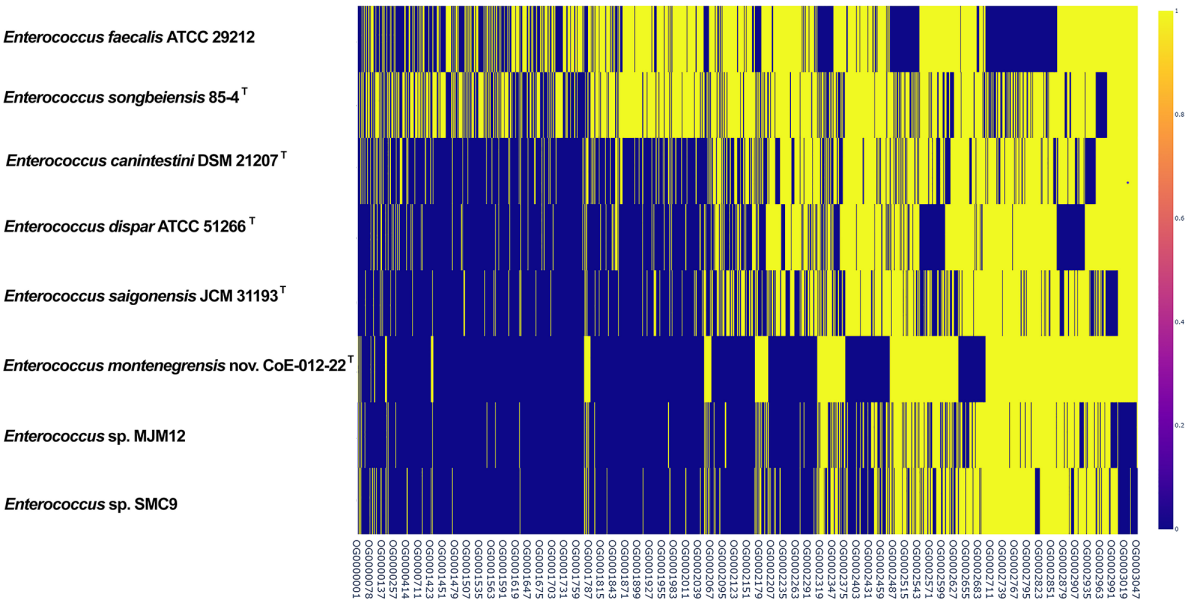

Fig. 3. Heatmap of orthofinder analysis with *E. montenegrensis* CoE-012-22^{T} and different enterococci strains included in this study. Genes are displayed as horizontal lines across samples (columns).

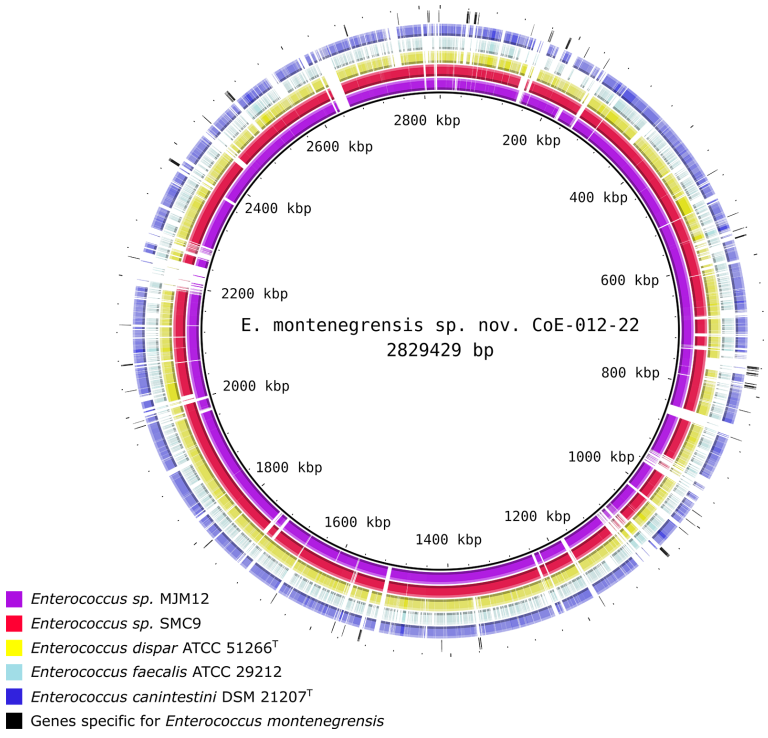

Fig. 4. Ring created with BRIG (version 0.95-dev.0003). *E. montenegrensis* CoE-012-22^{T} was compared with the two closely related strains *Enterococcus* sp. SMC-9 and *Enterococcus* sp. MJM12, *E. canintestini* DSM 21207^{T}, *E. dispar* ATCC 51266^{T} and *E. faecalis* ATCC 29212. Specific orthologous groups are shown – orthogroups are mentioned here as specific if they occur in *E. montenegrensis* CoE-012-22^{T} and in one of the comparison species other than *Enterococcus* sp. SMC-9 and *Enterococcus* sp. MJM12.

Table 2. Information on genes, functions and products found in strain E. montenegrensis CoE-012-22^T but absent in *Enterococcus* sp. MJM12, *Enterococcus* sp. SMC-9 and other enterococci

Gene	Function	Product
GO:0016788	Hydrolase activity, acting on ester bonds	NUMOD4 domain-containing protein
GO:0015144	Carbohydrate transmembrane transporter activity	Alpha-glucoside-specific PTS transporter subunit IIBC
GO:0009025	Tagatose-bisphosphate aldolase activity	Tagatose-bisphosphate aldolase
GO:0008747	*N*-Acetylneuraminate lyase activity	*N*-Acetylneuraminate lyase
GO:0003700	DNA-binding transcription factor activity	MarR family transcriptional regulator
GO:0010181	FMN binding	Flavodoxin
GO:0008982	Protein-N(PI)-phosphohistidine-sugar phosphotransferase activity	PTS cellobiose transporter subunit IIC

CoE-012-22^T was Gram-positive, facultative anaerobic and catalase- and oxidase-negative. Growth in M17 broth, BHI, Brain heart infusion broth, nutrient broth and on blood agar was determined at 37°C for 24 h. On blood agar supplemented with 5% sheep blood, colonies are small, whitish, smooth and circular. Growth in M17 broth (Difco), BHI broth and nutrient broth at different temperatures (4, 10, 45, 50°C), different NaCl concentrations (0–6.5%, w/v) at 37°C and different pH values (pH 3, 4, 5, 6, 7, 8 and 9.6) at 37°C was measured for 24 and 48 h. M17 broth (Difco) was used to determine the growth of *E. montenegrensis* CoE-012-22^T at different pH values. After autoclaving and cooling the pH was adjusted with NaOH and HCl. *E. montenegrensis* CoE-012-22^T was inoculated in triplicates at the different pH values. Survival of *E. montenegrensis* CoE-012-22^T at 60°C was examined by incubation of suspended cells in BHI, M17 and nutrient broth for 15, 30 and 60 min and subsequent incubation at 37°C for 24 and 48 h. Cells showed growth at 4–50°C, pH 5–8, with 6.5% (w/v) NaCl and survived 60°C in BHI and nutrient broth for 30 min. Optimum growth was monitored at 45°C in M17 and BHI broth (Table S3), and at a pH of 7.0–8.0 (Table S4). The growth with the addition of 6.5% (w/v) NaCl was much more significant compared to the growth without the addition of NaCl in the medium. The intensity of growth was evaluated based on the intensity of turbidity in the test tubes.

Semiquantification of enzyme activities, assimilation and acid production were determined using API ZYM, API STREP and API RAPID ID 32 STREP kits following the instructions of the manufacturer (bioMérieux). Strain CoE-012-22^T tested positive for esterase lipase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, acetonin production, *β*-glucosidase hydrolysis, pirrolidonyl arylamidase, leucine aminopeptidase, arginine dihydrolase, ribose, lactose, trehalose, starch and cyclodextrin; and tested negative for alkaline phosphatase, lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, α-glucosidase, α-mannosidase, α-fucosidase, arabinose (acidification), mannitol (acidification), sorbitol (acidification), inulin (acidification), raffinose (acidification), glycogen (acidification), sucrose (acidification), D-arabitol (acidification), alanyl-phenylalalanyl-proline arylamidase, hydrolysis of hippurate, pullulane (acidification), melezitose (acidification) and *β*-mannosidase (Table 3).

The enzyme activity profile differed from that of other *Enterococcus* species. For example, *E. montenegrensis* CoE-012-22^T was negative for alkaline phosphatase while *E. saigonensis* JCM 31193 ^{T}, *E. canintestini* DSM 21207 ^{T}, *E. dispar* ATCC 51266 ^{T} and *E. devriesei* DSM 22802^T were positive; *E. montenegrensis* CoE-012-22^T was negative for *β*-galactosidase, while *E. canintestini* DSM 21207^T, *E. dispar* ATCC 51266^T and *E. devriesei* DSM 22802^T were positive; *E. montenegrensis* CoE-012-22^T was negative for α-glucosidase while *E. saigonensis* JCM 31193^T, *E. devriesei* DSfM 22802^T and *E. faecalis* ATCC 29212 were positive; *E.*

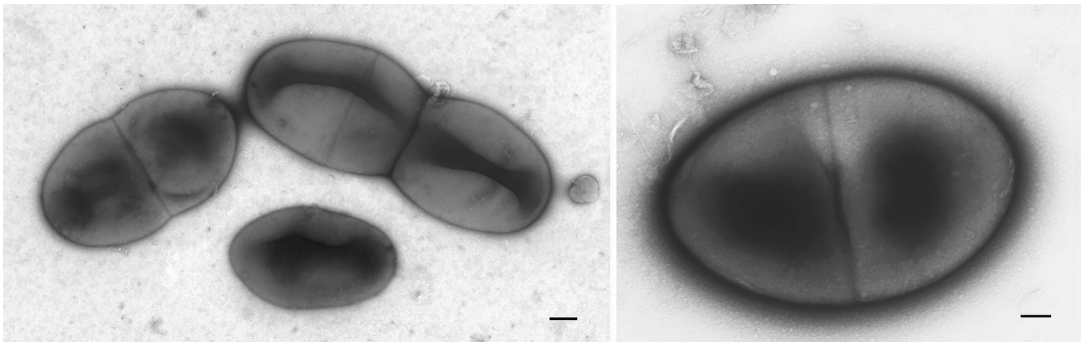

Fig. 5. Transmission electron microscopy image of *E. montenegrensis* CoE-012-22^T cells. Negative staining bar, 200 nm.

Table 3. Biochemical tests carried out using API ZYM, API STREP and API RAPID ID 32 STREP (bioMérieux) for Enterococcus montenegrensis CoE-012-22^T and the other enterococci type strains

Test reactions block 1 (API ZYM), block 2 (API STREP), block 3 (API RAPID ID 32 STREP). The following reactions were common for API ZYM, API STREP and API RAPID ID 32 STREP (therefore only reported once; same results were displayed with different kits): alkaline phosphatase, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, *β*-glucosidase, N-acetyl-*β*-glucosaminidase, arginine dihydrolase, acetonin production, ribose, mannitol, sorbitol, lactose, trehalose, raffinose and glycogen. +, Positive; –, negative

	Test	Enterococcus montenegrensis CoE-012-22^T	Enterococcus saigonensis JCM 31193^T	Enterococcus canintestini DSM 21207^T	Enterrococcus dispar ATCC 51266^T	Enterococcus devriesei DSM 22802^T	Enterococcus faecalis ATCC 29212
	Alkaline phosphatase	–	+	+	+	+	–
	Esterase lipase	+	+	+	+	+	+
	Lipase	–	–	–	–	–	–
	Leucine arylamidase	+	+	+	+	+	+
	Valine arylamidase	–	–	–	–	–	–
	Cystine arylamidase	–	–	–	–	–	–
	Trypsin	–	–	–	–	–	–
	α-chymotrypsin	–	+	–	–	–	–
1	Acid phosphatase	+	+	+	+	+	+
	Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	+
	α-galactosidase	–	–	–	–	–	–
	β-galactosidase	–	–	+	+	+	–
	β-glucuronidase	–	–	–	–	–	–
	α-glucosidase	–	+	–	–	+	+
	β-glucosidase	+	+	+	+	–	+
	N-acetyl-*β*-glucosaminidase	+	–	+	–	–	–
	α-mannosidase	–	–	–	–	–	–
	α-fucosidase	–	–	–	–	–	–
	Acetonin production	+	+	–	+	+	+
	Aesculin (*β*-Glucosidase hydrolisis)	+	+	+	+	+	+
	Pyrrolidonyl arylamidase	+	–	+	–	–	+
	Lucine aminopeptidase	+	–	–	–	–	+
	Arginine dihydrolase	+	+	+	+	+	+
	Ribose (acidification)	+	–	–	+	+	+
	Arabinose (acidification)	–	–	–	–	–	–
2	Mannitol (acidification)	–	–	–	–	+	+
	Sorbitol (acidification)	–	–	–	–	+	+
	Lactose (acidification)	+	+	–	+	+	–
	Trehalose (acidification)	+	+	–	+	+	+
	Inulin (acidification)	–	–	–	–	–	–
	Raffinose (acidification)	–	–	–	–	–	–
	Starch (acidification)	+	–	–	–	–	+

Continued

Table 3. Continued

	Test	Enterococcus montenegrensis CoE-012-22^T	Enterococcus saigonensis JCM 31193^T	Enterococcus canintestini DSM 21207^T	Enterrococcus dispar ATCC 51266^T	Enterococcus devriesei DSM 22802^T	Enterococcus faecalis ATCC 29212
	Glycogen (acidification)	–	–	–	–	–	–
	Sucrose (acidification)	–	–	+	–	+	+
	D-arabitol (acidification)	–	–	–	–	–	-
	Cyclodextrin (acidification)	+	+	–	–	–	+
	Alanyl-Phenylalalanyl-Proline Arylamidase	–	–	–	–	+	-
	Pyroglutamic acid Arylamidase	+	+	+	+	–	+
	Glycyl-Tryptophan Arylamidase	+	+	+	+	+	+
	Hydrolysis of hippurate	–	–	–	–	–	+
3	Pullulane (acidification)	–	–	–	–	–	–
	Maltose (acidification)	+	+	+	+	+	+
	Mellibiose (acidification)	+	+	–	–	–	–
	Melezitose (acidification)	–	–	–	–	+	+
	Methyl-ßD Glucopyranoside (acidification)	+	+	–	+	+	+
	Tagatose (acidification)	+	+	+	+	–	+
	ß-mannosidase	–	–	–	–	–	–
	Urease	+	+	+	+	+	–

montenegrensis CoE-012-22^T is positive for *N*-acetyl-*β*-glucosaminidase while the other enterococci but *E. canintestini* DSM 21207^T were negative, among other differences Table 3.

Antibiotic susceptibility testing against vancomycin and tetracycline was performed by E-test (bioMérieux) revealing that the strain was susceptible to vancomycin (0.38 μg ml^{-1}) and tetracycline (0.094 μg ml^{-1}).

CHEMOTAXONOMIC CHARACTERIZATION

Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on a Microflex LT/SH with database MBT Compass IVD 4.2 was used according to the manufacturer's instructions. Spectra were analysed using Flex Analysis version 3.4 (Bruker). MALDI-TOF identified the strain CoE-012-22^T as *E. canintestini* (log score 2). However, the mass spectrum of strain CoE-012-22^T differed from *E. canintestini* due to the lack of peaks at mass to charge ratios (m/z) 3420–3500, 4450–5300 and 7000–7900. A peak at m/z 10000 was only observed in the novel strain (Figs S3A and S3B). *E. dispar* ATCC 51266^T differed from CoE-012-22^T by the presence of peaks at m/z 3616, 4775, 7234 and 9552, which were absent in CoE-012-22^T. Instead, CoE-012-22^T had peaks at m/z 2653, 5312 and 6825, which were absent in *E. dispar* ATCC 51266^T (Figs S3A and S3C). MALDI-TOF spectra from 12 colonies of *E. montenegrensis* CoE-012-22^T gave identical profiles (Fig. S4).

The BAGEL4 [55] database was used to detect bacteriocins and antiSMASH 6.0 [56] to detect secondary metabolite biosynthetic gene clusters. Strain CoE-012-22^T carried the *subtilisin A* gene (a sactipeptide), and the cyclic lactone autoinducer. *Enterococcus* sp. MJM12 and *Enterococcus* sp. SMC-9 carried sactipeptides, *Blpk* and RiPP-like genes (Table S2). *E. canintestini* DSM 21207^T carried genes *Divercin_V41*, *enterocin A* and cyclic lactone autoinducer. *E. dispar* ATCC 51266^T and *E. saigonensis* JCM 31193^T carried no bacteriocin and no secondary metabolite genes.

The gene subtilisin A is one of the best characterized sactipeptide in enterococci. It was first isolated from *Bacillus subtilis* [57] revealing that it is a serine protease whose replacements of no less than 83 aminoacid residue positions may promote thermostability which would help the bacteria to survive at high temperatures and adverse circumstances. This property might be especially important in bacteria coming from food since they should adapt to adverse and extreme environments due to the

production methods to obtain the final product. In addition, the protein subtilisin A displays a broad spectrum activity against diverse bacteria [58], which is especially attractive in the context of food microbiology.

DESCRIPTION OF *ENTEROCOCCUS MONTENEGRENSIS* SP. NOV.

Enterococcus montenegrensis (mon.te.ne.gren'sis. M.L. masc. adj. *montenegrensis*, pertaining to Montenegro).

Cells are Gram-reaction-positive, non-motile, coccoid, approximately 1.8 µm long and 0.7 µm wide, arranged in pairs or short chains, and grow under aerobic and anaerobic conditions. Colonies are small, circular, regular, flat and white coloured. Growth occurs at 4–50°C (optimum at 45°C) and pH 6–8 (optimum at pH 7.0–8.0). Cells can tolerate up to 6.5% (w/v) NaCl and survive at 60°C for 30 min.

The species is positive for esterase lipase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucosidase, *N*-acetyl-β-glucosaminidase, acetonin production, β-glucosidase hydrolysis, pirrolidonyl arylamidase, leucine aminopeptidase, arginine dihydrolase, ribose, lactose, trehalose, starch and cyclodextrin; and tested negative for alkaline phosphatase, lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase, α-fucosidase, arabinose (acidification), mannitol (acidification), sorbitol (acidification), inulin (acidification), raffinose (acidification), glycogen (acidification), sucrose (acidification), D-arabitol (acidification), alanyl-phenylalalanyl-proline arylamidase, hydrolysis of hippurate, pullulane (acidification), melezitose (acidification) and β-mannosidase.

Strain CoE-012-22^T (DSM 115843^T=NCIMB 15468^T) was isolated from dried beef sausage (Govedii Kulen) from Montenegro in 2022 (42.442574°, 19.268646°). The genome size of the type strain is 2.80 Mb and the genomic DNA G+C content is 37.4mol%. The genome sequence DDBJ/ENA/GenBank accession number is CP120467, for the 16S rRNA gene sequence it is OQ627393 and for the raw sequence reads they are SRR23759723 and SRR23693802.

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

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