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Streptococcus rubneri sp. nov., isolated from the human throat

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The GenBank accession numbers for the 16S rRNA sequences of strains LMG 27205, LMG 27206, LMG 27207^T and MRI-F 18 are JX861483-JX861486. The EMBL accession numbers for the *pheS*, *atpA* and *rpoA* gene sequences are HE994080 through HE994106.

Novel, Gram-positive, ovoid lactic acid bacterial isolates LMG 27205, LMG 27206, LMG 27207^T and MRI-F 18 were obtained from throat samples of healthy humans. 16S rRNA gene sequence analyses indicated that these isolates belong to the genus *Streptococcus,* specifically the *Streptococcus mitis* group, with *Streptococcus australis* and *Streptococcus mitis* as nearest neighbours (99.45% and 98.56% 16S rRNA gene sequence similarity, respectively). Genotypic fingerprinting by AFLP and PFGE, DNA-DNA hybridisations, comparative sequence analysis of *pheS*, *rpoA*, *atpA* and physiological and biochemical tests revealed that these bacteria formed a taxon well-separated from its nearest neighbours and other established *Streptococcus* species and therefore represent a new species, for which the name *Streptococcus rubneri* sp. nov. is proposed (with LMG 27207^T = DSM 26920^T) as type strain.

The streptococci are lactic acid bacteria (LAB) which belong to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* and the family *Streptococcaceae*. Comparative 16S rRNA gene sequence analyses clusters *Streptococcus* species into six main species groups (Bentley *et al.,* 1991; Kawamura *et al.,* 1995) that are referred to as the *anginosus*, *bovis*, *mitis*, *mutans, pyogenes* and *salivarius* species groups (Kawamura *et al.,* 1995). The *mitis* group currently includes *S. australis, S. cristatus, S. gordonii, S. infantis, S. lactarius, S. massiliensis, S. mitis*, *S. oligofermentans*, *S. oralis, S. parasanguinis*, *S. peroris, S. pseudopneumoniae, S. pneumoniae*, *S. sanguinis*, *S. sinensis* and *S. tigurinus* (Kawamura *et al.,* 1999; Hoshino *et al.,* 2005; Glazunova *et al.*, 2006; Naser, 2006; Martín *et al*., 2011; Zbinden *et al.*, 2012), and combines the *S. mitis* and *S. sanguinis* groups reported by Facklam (2002). However, assignment of *S. massiliensis* to the *mitis* group is based on partial sequences of housekeeping genes, and not on a 16S rRNA gene sequence comparison which allocates it to the *mutans* group (Glazunova *et al*., 2006, 2010).

The isolates LMG 27205, LMG 27206, LMG 27207^T and MRI-F 18 were obtained during an investigation of the microbial populations associated with the throat of healthy human volunteers taking part in a probiotics study (Seifert *et al*., 2011). Isolates LMG 27205

and MRI-F 18 were taken from throat swabs of the same individual at different sampling occasions, while LMG 27206 and LMG 27207^T originated from throat swabs of different individuals participating in the study. Streptococci were isolated on Columbia CNA 5% sheep blood agar (Becton Dickinson, Heidelberg, Germany) and were purified by repeated streaking using the same medium.

The nearly complete 16S rRNA gene sequences of all four isolates were determined as described below. DNA was extracted according to the method of Pitcher *et al.* (1989), as modified for Gram-positive bacteria as described by Björkroth and Korkeala (1996). PCR products were purified and commercially sequenced at GATC Biotech (Constance, Germany) as described previously (Kostinek *et al.*, 2005). The four isolates had nearly identical 16S rRNA gene sequences (99.79 % similarity). The similarity calculation of the 16S rRNA gene sequence of strain LMG 27207^T towards related strains was done using EzTaxon (Kim *et al.,* 2012) and revealed the highest similarity to the corresponding gene of the *S. australis* and *S. mitis* type strains (sequence similarities of 99.45 % and 98.56 %, respectively).

The mothur software package v.1.28.0 (Schloss et al., 2009) and the corresponding SILVA SSURef 102 database (Pruesse et al., 2007) were used to align and trim the almost complete 16S rRNA gene sequence of strain LMG 27207^T (1457bp) and sequences of type strains of all established species of the *Streptococcus mitis* group. These sequences (1331bp) were imported into the software package MEGA (Molecular Evolutionary Genetics Analysis) version 5.10 (Tamura et al., 2011) and the evolutionary history was inferred by using the maximum-likelihood method based on the Tamura 3-parameter model. The tree with the highest log likelihood is shown in Figure **1**. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. A discrete gamma distribution was used to model evolutionary rate differences among sites and allowed for some sites to be evolutionarily invariable. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Sequence similarity calculations determined using the

mothur software package, indicated that the closest relatives of strain LMG 27207^T is *Streptococcus australis* ATCC700641^T (99.4%). Lower sequence similarities (<98.6%) were found towards other species of the *Streptococcus mitis* group.

In order to study the relatedness between the four isolates and their nearest phylogenetic neighbours in more detail, fluorescent amplified fragment length polymorphism (FAFLP) and pulsed field gel electrophoresis (PFGE) of genomic DNA were performed. FAFLP fingerprinting of whole genomes was performed as described previously (Sistek *et al*., 2012). The resulting electrophoretic patterns were analysed using the Gene Mapper 4.0 software package (Applera Co.), and normalised tables of peaks were transferred into the BioNumerics version 5.1 software package (Applied Maths, Sint-Martens-Latem, Belgium). The FAFLP fingerprints of LMG 27205, LMG 27206, LMG 27207^T and MRI-F 18 formed a single cluster, well separated from fingerprints of *S. australis* and *S. parasanguinis* strains which were the nearest neighbours, and from other species belonging to the *S. mitis* group (Fig 2). In addition, the cluster showed three distinct DNA fingerprint types (with LMG 27205 and MRI-F 18 forming a single type), suggesting that LMG 27205, LMG 27206, LMG 27207^T and MRI-F 18 represent at least three different strains.

PFGE using the restriction enzyme *Sma*I was additionally used to investigate the clonality of the four isolates. PFGE was done as described by Huch *et al.* (2008). The fingerprints again showed that LMG 27205 and MRI-F 18 had highly similar fingerprints, while LMG 27206 and LMG 27207^T showed distinct fingerprints (Fig. S1). Together, the PFGE and AFLP results showed that LMG 27205 and MRI-F 18, which were isolated from the same individual at different occasions, appear to be clonally related and most probably represent re-isolates of the same strain.

Alternative marker genes such as the superoxide dismutase gene (*sodA*) (Glazunova *et al*., 2006; Poyart *et al.,* 1998, 2002), the endoribonuclease P gene (Täpp *et al.,* 2003), *recN* (Glazunova *et al*., 2010) and the genes encoding the alpha subunits of phenylalanyl-tRNA synthase (*pheS*), RNA polymerase (*rpoA*) and ATP synthase (*atpA*) have been used as tools with a high discriminatory power for species delineation of streptococci and other LAB. In the

present study, we used the primers listed in Table S1 for the amplification and sequencing of the *pheS*, *atpA* and *rpoA* genes as described by Naser (2006). The primer combinations pheS-21-F/pheS-23-R, rpoA-21-F/rpoA-23-R and atpA-20-F/atpA-26-R amplified the target genes of most strains. Where necessary, an alternative primer combination for *rpoA* (rpoA-20-F/rpoA-22-R) and *atpA* (atpA-20-F/atpA-26-R) was used. Amplification conditions and sequencing reactions were performed as described by Naser *et al.* (2005a; 2005b). SeaView version 4 was used to concatenate the *pheS*, *rpoA,* and *atpA* gene sequences of all four isolates and of those of *S. parasanguinis, S. australis* and *S. mitis* reference strains (Gouy *et al.*, 2010). The software package MEGA (Molecular Evolutionary Genetics Analysis) version 5.0 (Tamura *et al.*, 2011) was used to align the translated concatenated gene sequences and to analyse the nucleotide sequences. The statistical reliability of tree topologies was evaluated by bootstrapping analysis based on 1000 tree replicates. The neighbour-joining and the maximum-parsimony trees revealed topologies (data not shown) similar to those obtained in a phylogenetic tree constructed following the maximum-likelihood approach (Fig. 3). Pairwise sequence similarity calculations of the concatenated *pheS*, *rpoA,* and *atpA* gene sequences demonstrated that the taxon represented by the isolates LMG 27205, LMG 27206, LMG 27207^T and MRI-F 18 was readily distinguished from its nearest neighbours; the closest relatives of strain LMG 27207^T was *S. parasanguinis* LMG 14537^T with which it shared 98% concatenated sequence similarity. Lower sequence similarities were found (< 90.1%) towards the remaining *Streptococcus* species (data not shown).

DNA-DNA hybridisations were performed between strain LMG 27207^T and the type strains of *S. australis* (LMG 21714^T) and *S. parasanguinis* (LMG 14537^T). DNA for these hybridisation experiments and for the determination of the DNA base composition was extracted and purified using the method described by Gevers *et al*. (2001) with minor modifications (use of proteinase K after enzymatic lysis, of a 10 mM Tris-25 mM EDTA buffer to dissolve the extracted DNA, and of an additional DNA extraction step after RNase treatment). DNA-DNA hybridisations (four replications) were performed in the presence of 50 % formamide at 37°C using a modification (Goris *et al*., 1998; Cleenwerck *et al*., 2002) of the

microplate method described by Ezaki *et al*. (1989). Reciprocal reactions (A x B and B x A) were performed for each DNA pair and their variation was within the limits of this method (Goris *et al*., 1998). Strain LMG 27207^T exhibited DNA-DNA relatedness values below the species level (< 70 %; Wayne *et al.*, 1987; Stackebrandt *et al*., 2002) towards *S. australis* LMG 21714^T (54 ± 5.5 % standard deviation) and *S. parasanguinis* LMG 14537^T (38 ± 0.5 % standard deviation), confirming that it indeed represents a novel species.

The DNA base composition of LMG 27207^T was determined by HPLC as described by Mesbah *et al*. (1989) using a Waters Breeze HPLC system and XBridge Shield RP18 column. The solvent used was 0.02M $NH_4H_2PO_4$ (pH 4.0) 1.5% (v/v) acetonitrile. Non-methylated lambda phage (Sigma, Taufkirchen, Germany) and *E. coli* DNA were used as calibration reference and control, respectively. The G+C content of strain LMG 27207^T was 40.9 mol%.

Selected phenotypic characteristics of the novel taxon represented by strains LMG 27205, LMG 27206, LMG 27207^T and other streptococci belonging to the *mitis* group are shown in Table 1. The novel taxon can be distinguished from all or most other members of this group by its β-haemolytic activity on Columbia 5% sheep blood agar and raffinose utilisation, respectively. Furthermore, none of the species of this group can ferment melibiose except for *S. parasanguinis* (Martín *et al.,* 2011) and the isolates LMG 27205 and MRI-F 18. Apart from these traits there are no further phenotypic characteristics of the novel taxon that allow clear discrimination of the novel taxon from the other members of the *mitis* group (see Table 1).

In conclusion, the results of this polyphasic study demonstrate that the strains LMG 27205, LMG 27206, LMG 27207^T and MRI-F 18 represent a novel *Streptococcus* species that can be distinguished both genotypically and phenotypically from its nearest phylogenetic neighbours. We propose to classify these microorganisms as *Streptococcus rubneri* sp. nov., with strain LMG 27207^T (=DSM 26920^T) being the type strain.

Description of *Streptococcus rubneri* sp. nov.

Streptococcus rubneri (rub'ne.ri N.L. masc. gen n. *rubneri*) in honour of Max Rubner, 2 June 1854 – 27 April 1932, a German medical doctor and professor for hygiene, after which the Max Rubner-Institut was named and where the strains investigated in this study were isolated in Karlsruhe, Germany. Cells are Gram-positive, non-spore-forming cocci. Colonies grown on Columbia CNA 5% sheep blood agar at 37°C had a size of 1-3 mm in diameter, raised with an entire margin. The colour was opaque white and the zone of β-hemolysis was ca. 2 mm.

All strains grow in brain heart broth at 25, 30 and 37°C, while only isolates LMG 27205 and MRI-F 18 grow in this medium at 45°C. None of the strains produce gas in brain heart medium and none grow at pH 9.6. The bacteria are facultatively anaerobic and catalase-negative. Acetoin is not produced from glucose (VP test). The strains do not hydrolyse arginine, esculin, or hippurate or starch and are negative in tests for pyrrolidonyl arylamidase, α-D-galactosidase, β-D-galactosidase, β-D-glucosidase, β-D-glucuronidase and alkaline phosphatase. All strains were positive for leucine amino peptidase. None of the strains produced acid from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, β-methyl- D-xyloside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, methyl-α D-mannopyranoside, methyl-α D-glucopyranoside, amygdalin, arbutin, esculin, D-trehalose, inulin, D-melezitose, amidon, glycogen, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluonate, and 5-ketogluconate. Utilisation of D-sorbitol and D-melibiose are variable, with the type strain LMG 27207^T being negative for both. Strain LMG 27206 was the only isolate capable of D-sorbitol utilisation and showed a weak positive reaction for production of acid from D-melibiose. Strains MRI-F18 and LMG 27205 were positive for D-melibiose utilisation. All strains were capable of acid production from D-galactose, D-glucose, D-fructose, D-mannose, N-acetyl-glucosamine, salicin, D-cellobiose, D-maltose, D-lactose, D-sucrose and D-raffinose.

The type strain, LMG 27207^T (= DSM 26920^T), was isolated from a human throat in Germany in 2007 and has a DNA G+C content of 40.9 mol%.

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LEGENDS TO FIGURES

Figure 1: Molecular phylogenetic analysis of the 16S rRNA gene by the maximum likelihood method based on the Tamura 3-parameter mode. Bootstrap values (%) based on 1000 replications are shown at the branch points. The bar indicates 1% sequence divergence.

Figure 2: AFLP DNA fingerprints of *Streptococcus rubneri* sp. nov. and of strains of species of the *Streptococcus mitis* group. The dendrogram derived from unweighted pair-group cluster analysis (UPGMA) of the fingerprints with levels of linkage expressed as DICE similarity coefficients.

Figure 3: Concatenated tree based on *pheS*, *rpoA* and *atpA* gene sequences of *Streptococcus* strains (1377 bp). Distance estimations were obtained by the Jukes and Cantor model. Bootstrap percentages (>50) after 1000 simulations are shown.

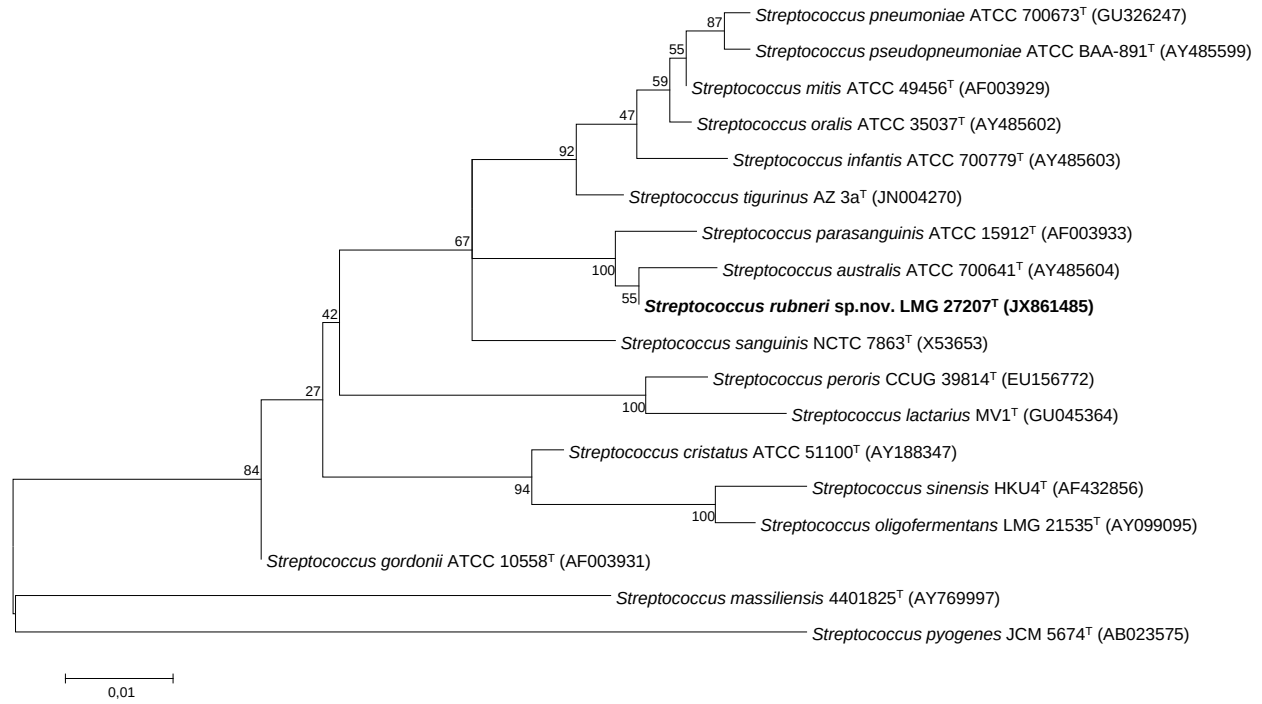
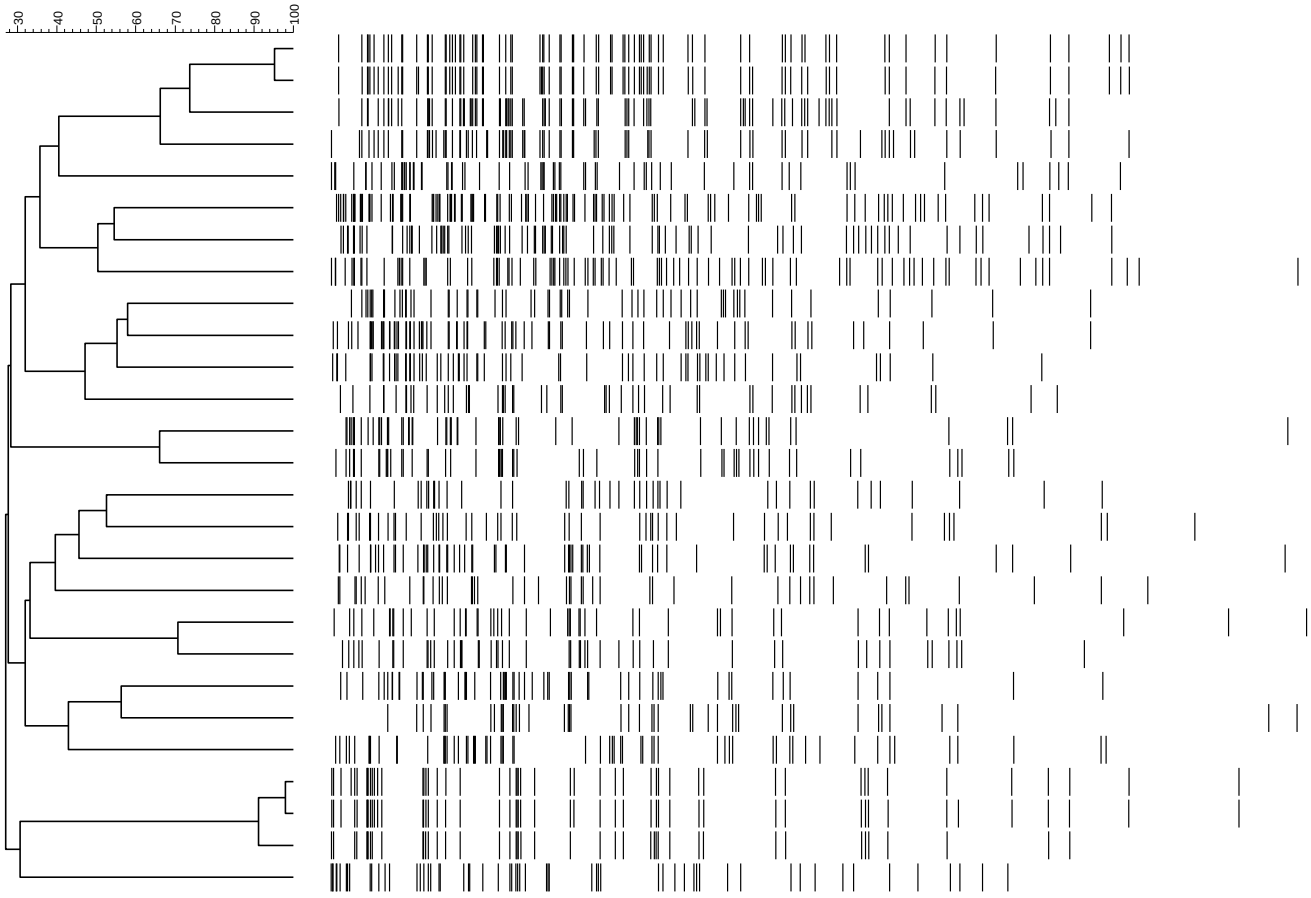

87
55
59
47
92
67
100
55
42
27
100
84
94
100
Streptococcus pneumoniae ATCC 700673T (GU326247)
Streptococcus pseudopneumoniae ATCC BAA-891T (AY485599)
Streptococcus mitis ATCC 49456T (AF003929)
Streptococcus oralis ATCC 35037T (AY485602)
Streptococcus infantis ATCC 700779T (AY485603)
Streptococcus tigurinus AZ 3aT (JN004270)
Streptococcus parasanguinis ATCC 15912T (AF003933)
Streptococcus australis ATCC 700641T (AY485604)
Streptococcus rubneri sp.nov. LMG 27207T (JX861485)
Streptococcus sanguinis NCTC 7863T (X53653)
Streptococcus peroris CCUG 39814T (EU156772)
Streptococcus lactarius MV1T (GU045364)
Streptococcus cristatus ATCC 51100T (AY188347)
Streptococcus sinensis HKU4T (AF432856)
Streptococcus oligofermentans LMG 21535T (AY099095)
Streptococcus gordonii ATCC 10558T (AF003931)
Streptococcus massiliensis 4401825T (AY769997)
Streptococcus pyogenes JCM 5674T (AB023575)
0,01

Fig. 1



***S. rubneri* sp. nov.**	**MRI-F 18**
***S. rubneri* sp. nov.**	**LMG 27205**
***S. rubneri* sp. nov.**	**LMG 27206**
***S. rubneri* sp. nov.**	**LMG 27207^{T}**
S. australis	LMG 21762^{T}
S. parasanguinis	LMG 14539
S. parasanguinis	LMG 17595
S. parasanguinis	LMG 14537^{T}
S. sanguinis	LMG 14657
S. sanguinis	LMG 14702^{T}
S. sanguinis	LMG 14513
S. sanguinis	LMG 14639
S. peroris	LMG 18719^{T}
S. peroris	LMG 25209
S. oralis	LMG 14535
S. oralis	LMG 14533
S. oralis	LMG 14532^{T}
S. oralis	LMG 17851
S. pneumoniae	LMG 14545^{T}
S. pneumoniae	LMG 21598
S. mitis	LMG 14555
S. mitis	LMG 14557^{T}
S. mitis	LMG 14552
S. oligofermentans	LMG 22276
S. oligofermentans	LMG 22277
S. oligofermentans	LMG 22279^{T}
S. infantis	LMG 18720^{T}

Fig. 2

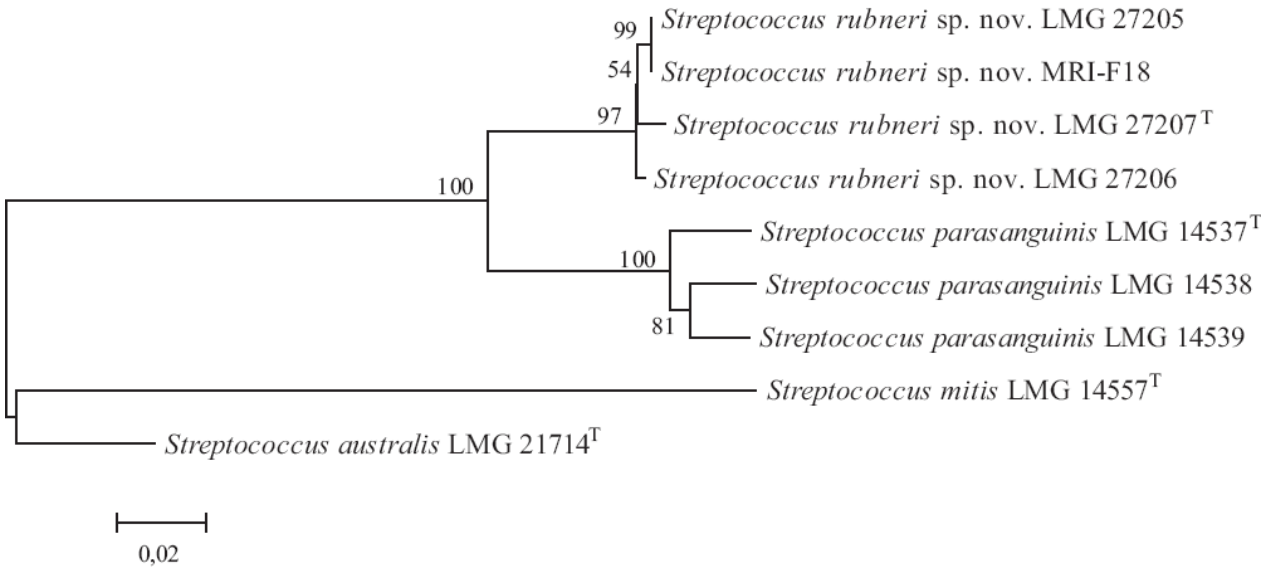

99
54
97
100
100
81
Streptococcus rubneri sp. nov. LMG 27205
Streptococcus rubneri sp. nov. MRI-F18
Streptococcus rubneri sp. nov. LMG 27207T
Streptococcus rubneri sp. nov. LMG 27206
Streptococcus parasanguinis LMG 14537T
Streptococcus parasanguinis LMG 14538
Streptococcus parasanguinis LMG 14539
Streptococcus mitis LMG 14557T
Streptococcus australis LMG 21714T
0,02

Fig. 3

Table 1: Biochemical characteristics that differentiate the novel *S. rubneri* strains from other members of the *mitis* group. Strains: 1, *S. australis* (Willcox et al. 2001); 2, *S. cristatus* (Handley et al., 1991; Willcox et al. 2001); 3, *S. gordonii* (Kilian et al., 1989; Willcox et al. 2001); 4, *S. infantis* (Kawamura et al., 1998; Whiley and Hardie, 2009); 5, *S. lactarius* (Martín et al., 2011); 6, *S. massiliensis* (Glazunova et al., 2006); 7, *S. mitis* (Whiley and Hardie, 2009); 8, *S. oligofermentans* (Tong et al., 2003); 9, *S. oralis* (Bridge and Sneath, 1982; Whiley and Hardie, 2009); 10, *S. parasanguinis* (Whiley et al., 1990; Willcox et al. 2001); 11, *S. peroris* (Kawamura et al., 1998; Martín et al., 2011); 12, *S. pneumoniae* (Whiley and Hardie, 2009); 13, *S. pseudopneumoniae* (Arbique et al., 2004; Zbinden et al., 2012); 14, *S. rubneri* (this study); 15, *S. sanguinis* (Willcox et al. 2001); 16, *S. sinensis* (Woo et al., 2002); 17, *S. tigurinus* (Zbinden et al., 2012).

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
haemolysis	α	α	α	α	α	γ	α	α	α	α	α	α	α	β	α	α	α
acetoin (Vogues-Proskauer)	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
hydrolysis of:																	
arginine	+	d	+	-	+	+	d	-	-	+	-	+	-	-	+	+	-
esculin	ND	-	+	-	+	ND	-	-	d	d	-	d	-	-	d	+	-
hippurate	-	ND	-	-	+	+	-	+	-	ND	-	-	-	-	-	-	-
starch	ND	ND	d	ND	ND	-	d	ND	+	ND	ND	-	-	-	d	ND	+
production of:																	
α-D-galactosidase	-	-	-	-	-	-	+	d	-	+	-	+	-	-	-	-	-
β-D-galactosidase	-	-	-	+	-	-	-	-	+	+	-	+	+	-	d	-	+
β-D-glucosidase	-	-	-	-	+	-	-	-	-	-	-	-	d	-	-	-	-
acid from:																	
D-lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
D-mannitol	-	-	-	-	-	-	-	-	+	-	-	d	d	-	-	-	-
D-melibiose	-	-	-	-	-	-	d	-	d	+	-	--	-	d	-	-	-
D-raffinose	-	-	d	-	-	-	d	d	d	d	-	+	-	+	d	-	+
D-ribose	-	-	-	-	-	-	d	-	d	ND	-	-	-	-	-	-	-
D-sorbitol	-	+	-	-	-	-	-	-	-	d	-	-	-	d	d	-	-
D-sucrose	+	-	+	+	+	-	+	+	+	+	+	+	d	+	+	+	+
D-tagatose	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
D-trehalose	-	+	+	-	-	-	d	d	d	d	-	d	-	-	+	+	d

ND: not determined/reported; d, variable (For the *S. rubneri* type strain LMG 27207^T the utilization for D-sorbitol and D-melibiose was negative).

Supplementary figure S1

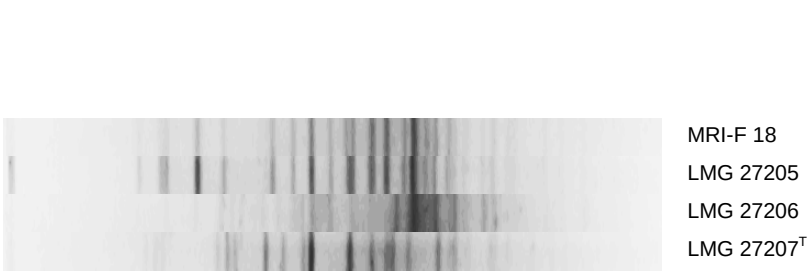


Figure legend S1:
Normalised *Sma*I PFGE profiles of the *Streptococcus rubneri* isolates LMG 27205, 27206, 27207^{T} and MRI-F 18 used for determining clonal relationships

Supplementary Table S1

Amplification and sequencing primers used in this study

Primer name	Sequence (5'→3')	Position
pheS-21-F	CAYCCNGCHCGYGAYATGC	557
pheS-23-F	GGRTGRACCATVCCNGCHCC	968
rpoA-20-F	ATGWTNGARWTWGAAAARCC	1
rpoA-21-F	ATGATYGARTTTGAAAAACC	1
rpoA-22-R	ACYTTVATCATNTCWGVYTC	844
rpoA-23-R	ACHGTRTTRATDCCDGCRCG	802
atpA-20-F	TAYRTYGGKGAYGGDATYGC	97
atpA-22-F	GCWCCYGGTRTYATGCARCG	397
atpA-23-R	CGYTGCATRAYACCRGGWGC	397
atpA-24-F	GATGAYYTWTCAAARCAAGC	781
atpA-25-R	GCTTGYTTTGAWARRTCATC	781
atpA-26-R	TTCATBGCYTTRATYTGNGC	1108