Taxonomic study of *Weissella confusa* and description of *Weissella cibaria* sp. nov., detected in food and clinical samples

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A taxonomic study was conducted to clarify the relationships of two bacterial populations belonging to the genus *Weissella*. A total of 39 strains originating mainly from Malaysian foods (22 strains) and clinical samples from humans (9 strains) and animals (6 strains) were analysed using a polyphasic taxonomic approach. The methods included classical phenotyping, whole-cell protein electrophoresis, 16S and 23S rDNA RFLP (ribotyping), determination of 16S rDNA sequence homologies and DNA–DNA reassociation levels. Based on the results, the strains were considered to represent two different species, *Weissella confusa* and a novel *Weissella* species, for which the name *Weissella cibaria* sp. nov. is proposed. *Weissella confusa* possessed the highest 16S rDNA sequence similarity to *Weissella cibaria*, but the DNA–DNA reassociation experiment showed hybridization levels below 49% between the strains studied. The numerical analyses of *Weissella confusa* and *Weissella cibaria* strains did not reveal any specific clustering with respect to the origin of the strains. Based on whole-cell protein electrophoresis, and *Claire* and *HindIII* ribotyping patterns, food and clinical isolates were randomly located in the two species-specific clusters obtained.

**Keywords:** *Weissella confusa*, *Weissella cibaria*, Malaysian foods, clinical samples

INTRODUCTION

The phylogeny of the bacteria classified currently in the genus *Weissella* was clarified in 1990. Using both 16S and 23S rRNA sequence data, Martinez-Murcia & Collins (1990) and Martinez-Murcia et al. (1993) showed that *Leuconostoc paramesenteroides* is phylogenetically distinct from *Leuconostoc mesenteroides* and that it groups together with five heterofermentative lactobacilli, *Lactobacillus confusus*, *Lactobacillus halotolerans*, *Lactobacillus kandleri*, *Lactobacillus minor* and *Lactobacillus viridescens*. In a study of leuconostoc-like organisms originating from fermented sausages (Collins et al., 1993) the taxonomy of these species was further assessed. This resulted in the description of the genus *Weissella* comprising the former *Leuconostoc paramesenteroides*, the five *Lactobacillus* species, and at that time a novel species, *Weissella hellenica*. Recently, a novel species, *Weissella thailandensis*, has been described and suggested to belong to this genus (Tanasupawat et al., 2000). Thus there are currently eight species in the genus *Weissella*, *Weissella confusa*, *Weissella halotolerans*, *Weissella hellenica*, *Weissella kandleri*, *Weissella minor*, *Weissella paramesenteroides*, *Weissella thailandensis* and *Weissella viridescens*.

*Weissella* strains have been isolated from a variety of sources. *Weissella paramesenteroides* is one of the predominant species in fresh vegetables and it also plays an important role in the first phase of silage fermentation (Dellaglio et al., 1984; Dellaglio & Torriani, 1986). *Weissella halotolerans*, *Weissella hellenica* and *Weissella viridescens* have been commonly associated with meat or meat products (Niven et al., 1957; Milbourne, 1983; Collins et al., 1993), whereas the natural habitat of *Weissella kandleri* is unknown.

**Abbreviation:** LAB, lactic acid bacteria.

The EMBL accession number for the 16S rRNA gene sequence of LMG 17699T is AJ295989.
Table 1. Strains used in the study

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<td>3507†</td>
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*BCCM/LMG, Belgian Coordinated Collections of Microorganisms, Laboratorium Microbiologisch Gent Culture Collection, Universiteit Gent, Belgium; CCUG, Culture Collection of the University of Göteborg, Department of Clinical Bacteriology, Göteborg, Sweden; DSM, German Collection of Micro-organisms and Cell Cultures, Braunschweig, Germany; NCFB, National Collection of Food Bacteria, Reading, UK; NCIB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK; NRRL, Agricultural Research Service Culture Collection. Peoria, IL, USA.
†L. A. Devriese, Ghent University, Belgium; E. Falsen, University of Göteborg, Sweden; H. Goossens, University of Antwerp, Belgium; J. Leisner, Royal Veterinary and Agricultural University, Frederiksberg, Denmark.
of Weissella cibaria was done using the strains LMG 17706, LMG 17699\textsuperscript{T}, LMG 18506, LMG 18507 and LMG 18814. Strains LMG 18477, LMG 17718 and the Weissella confusa type strain LMG 9497\textsuperscript{T} were selected to represent the Weissella confusa cluster. Production of ammonia from arginine was determined using the method of Briggs (1953). Dextran formation was studied on 5\% sucrose containing agar (Harrigan & McCance, 1976). The API 50 CHL Lactobacillus identification system (BioMérieux) was used for the determination of the carbohydrate fermentation profiles. The ability to produce different lactic acid isomers was tested using an enzymic method (Von Krush & Lompe, 1982) utilizing d- and l-lactate dehydrogenases (Roche). The eight strains were also tested for growth in MRS broth (Difco) incubated at 4, 15, 37 and 45 °C until growth was observed or otherwise at least for 21 d. Growth in the presence of 6.5 and 8.0 \% of NaCl was tested in MRS broth incubated at 30 °C until growth was observed or otherwise at least for 21 d.

Peptidoglycan analysis. Preparation of cell walls and determination of peptidoglycan structure of LMG 17699\textsuperscript{T} and LMG 18814 strains were carried out by the methods described by Schleifer & Kandler (1972) with the modification of using thin layer chromatography on cellulose sheets instead of paper chromatography. Briefly, 1 mg freeze-dried cell walls was hydrolysed in 0.2 ml 4 M HCl at 100 °C for 16 h (total hydrolysate) or 45 min (partial hydrolysate). Diamino acids were determined from total hydrolysate by one-dimensional chromatography in the solvent system methanol/pyridine/water (320:40:70:10 by vol.). Amino acids and peptides from total and partial hydrolysates were identified after two-dimensional chromatography in the systems described by Schleifer & Kandler (1972) according to mobility and staining characteristics with ninhydrin spray. The resulting ‘fingerprints’ were compared with known peptidoglycan structures.

Whole-cell protein analysis. Preparation of cellular protein extracts and PAGE was performed as described by Pot et al. (1994). Briefly, discontinuous gels were run overnight at constant current and temperature in a vertical slab apparatus. The separation gel was 12.6 cm long and contained 12\% constant acrylamide (the monomer solution contained 30\% total acrylamide with 2.67\% cross-linking in 0.375 M Tris/HCl, pH 8.8, and 0.1\% SDS); the stacking gel was 12 cm long and contained 5\% total acrylamide (the monomer solution contained 30\% total acrylamide with 2.67\% cross-linking in 0.125 M Tris/HCl, pH 6.8, and 0.1\% SDS). Protein bands were stained with Coomassie blue R-250 in 50\% (v/v) methanol and 10\% (v/v) acetic acid. These conditions allowed separation of proteins and peptides in the molecular mass range of 14000 to 116000.

Isolation of DNA, restriction endonuclease analysis, and 16S and 23S rDNA RFLP (ribotyping). ClaI, EcoRI and HindIII restriction enzymes (New England Biolabs) were used for ribotyping of all strains. DNA was isolated by the guanidium thiocyanate method of Pitcher et al. (1989), as modified by Björkroth & Korkeala (1996), by the combined lysozyme and mutanolysin (Sigma) treatment. Restriction endonuclease treatment of 3 μg DNA was done as specified by the manufacturer (New England Biolabs) and electrophoresis was carried out as described previously (Björkroth & Korkeala, 1996). Genomic blots were made using a vacuum device (VacuGene; Pharmacia) and the rDNA probe for

METHODS

Bacterial strains. Table 1 shows the strains used in this study. Depending on the purpose, the strains were grown either for 3 d on MRS agar (Oxoid) at 30 °C in a microaerophilic atmosphere (approx. 5\% O\textsubscript{2}, 10\% CO\textsubscript{2} and 85\% N\textsubscript{2}) or overnight in MRS broth (Difco) at 30 °C. All strains were maintained in MRS broth (Difco) at −70 °C.

Phenotypic characterization. All Weissella cibaria and Weissella confusa strains were Gram-stained, catalase-tested and studied for the production of gas from glucose (Smittle & Cirigliano, 1992). Further phenotypic characterization

In recent studies, when the lactic acid bacterium (LAB) populations associated with traditionally fermented foods have been characterized (Hancioglu & Karapinar, 1997; Ampe et al., 1999; Paludan-Müller et al., 1999) many of these foods have been found to contain Weissella species. The recently characterized species, Weissella thailandensis, was also isolated from fermented fish product in Thailand (Tanasupawat et al., 2000). In non-fermented foods from Southeast Asia, LAB have often been detected as contaminants (Leisner et al., 1997, 1999). Our study set out to identify some LAB originating mainly from the Malaysian foods tapai and chili bo. Tapai is sweet, fermented glutinous rice or cassava, and chili bo is non-fermented chili and cornstarch containing perishable food ingredients. Initially, when the protein profiles and ribotypes of the LAB strains from tapai and chili bo were analysed, the patterns showed similarity to the patterns of the Weissella confusa strains. However, further analysis showed that some of these strains formed a distinct cluster, separate from the cluster containing the Weissella confusa type strain. During this stage, some unidentified LAB strains originating from clinical samples were also detected joining both clusters. These findings warranted a taxonomic study clarifying the relationship of the LAB strains within these two groups.

The methods used in this polyphasic study included classical phenotyping, whole-cell protein electrophoresis, 16S and 23S rDNA RFLP analysis (Grimont & Grimont, 1986) and the determination of both 16S rDNA sequence and DNA–DNA reassocation levels. Based on the results, the LAB were considered to represent two different species, Weissella confusa and a novel Weissella species, for which we propose the name Weissella cibaria sp. nov.

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ribotyping was labelled by reverse transcription [AMV-RT (Promega) and Dig Labelling Kit (Roche)] as described by Blumberg et al. (1991). Membranes were hybridized at 68 °C as described by Björkroth & Korkeala (1996).

**Pattern analysis.** Whole-cell protein profiles were scanned using an LKB 2202 UltraScan Laser Densitometer (LKB). The densitometric analysis, normalization and interpolation of the protein profiles were performed using the GelCompar 4.2 software package (Applied Maths). Numerical analysis was performed using the BioNumerics 1.0 software package (Applied Maths). The similarity between all pairs of traces was expressed by the Pearson product moment correlation coefficient converted for convenience to a percentage value. For numerical analysis, the similarity between all pairs was performed using the BioNumerics 1.0 software package. The similarity between all pairs was expressed by the Dice coefficient correlation and UPGMA (unweighted pair group method using arithmetic averages) clustering was used for the construction of the dendrogram.

**16S rRNA gene sequence analysis.** Part of the rDNA operon, comprising the nearly complete 16S DNA, was amplified by PCR. The forward primer was 5’-CTGGCTCAAGGAYGAAACGCTG-3’, corresponding to positions 19–38 (Escherichia coli 16S rRNA numbering). The reverse primer was 5’-AAGGAGGTGATCCAGCCGCA-3’, complementary to positions 1541–1522. PCR-amplified 16S rDNAs were purified by using the QIAquick PCR Purification Kit (Qiagen). Sequence analysis was performed using an Applied Biosystems 377 DNA Sequencer and the protocols of the manufacturer (Perkin-Elmer, Applied Biosystems) using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (with AmpliTaq DNA polymerase). The sequencing primers were those given by Coenye et al. (1999). Sequence assembly was performed by using the program AutoAssembler (Applied Biosystems). Phylogenetic analysis of the 16S rDNA sequence of strain LMG 17699T was performed by using the GeneCompar 2.0 software package (Applied Maths). The consensus sequence and the sequences of strains belonging to the same phylogenetic group of Weisella (retrieved from the NCBI GenBank database) were aligned. The accession numbers of the 16S rDNA sequences used are: Weisella confusa LMG 9497T, M23036; Weisella halotolerans LMG 9469T, M23037; Weisella hellenica LMG 15125T, X95981; Weisella kandleri LMG 14471T, M23038; Weisella minor LMG 9847T, M23039; Weisella paramesenteroides LMG 9852T, M23033; Weisella viridescens LMG 3507T, M23040; Weisella thailandensis FS61-1T, AB023838; Leuconostoc gascomitatum LMG 18811T, AF231131. Calculation of the level of similarity and construction of a phylogenetic tree was based on the neighbour-joining method.

**DNA base composition and optical DNA–DNA hybridization analyses.** Table 2 shows the pairs selected for DNA–DNA hybridization tests. These organisms were selected based on the clustering observed in the numerical analyses of protein and ribotyping data. The large-scale DNA isolation was performed using the modified (Björkroth & Korkeala, 1996) guanidium thiocyanate method of Pitcher et al. (1989). The method was scaled up 10-fold and applied to cells from 200 ml of a well-grown MRS broth culture for each batch of isolation. DNA from one batch was dissolved overnight in 1 ml TE 10:1 (10 mM Tris, 1 mM EDTA, pH 8.0). Ribonuclease A (Sigma) was added to provide a concentration of 125 µg ml⁻¹ and the solution was incubated at 37 °C with gentle shaking for 1 h. Following the 1 h incubation, proteinase K (Sigma) was added to a concentration of 0.5 mg ml⁻¹ and incubation at 37 °C was continued at least for 6 h. DNA was precipitated as described by Pitcher et al. (1989) and dissolved in 1 ml 0.1 M SSC. When dissolved, the SSC concentration of a sample was adjusted with 20 × to 1 × SSC. Purified DNA was dialysed twice overnight at 4 °C using a 12000 to 14000 Da pore-size membrane (Medicell International). The first dialysis was carried out against 1 × SSC/EDTA (10 mM) and the second against 1 × SSC. DNA was fragmented two times in a French pressure cell press (SML Aminco; Colora Messtechnik) at about 1.5 × 10⁶ Pa. Before reassociation, it was dialysed once more overnight at 4 °C against 2 × SSC. The DNA base composition (mol% G+C) was estimated by the thermal denaturation method (De Ley et al., 1970) and the DNA homology values were determined from renaturation rates using a Gilford Response spectrophotometer (Ciba Corning Diagnostics).

**RESULTS AND DISCUSSION**

**Phenotypic characterization**

In basic phenotypic tests Weisella cibaria sp. nov. strains produced typical reactions for genus Weisella (Collins et al., 1993) and all phenotypic reactions of Weisella confusa strains LMG 18477, LMG 17718 and LMG 9497T were in agreement with the characteristics
Weisella cibaria strains LMG 9497<sup>T</sup>, LMG 17706, LMG 17699<sup>T</sup>, LMG 18506, LMG 18507 and LMG 18814 were Gram-positive rods, catalase-negative, grew at 15, 37 and 45 °C, and produced gas from glucose. None of the five strains grew at 4 °C, nor in the presence of 8% NaCl, but all grew at 6.5% NaCl. They all produced slime from sucrose, hydrolysed arginine and synthesized NaCl. They all produced slime from sucrose, hydrolysed arginine and synthesized NaCl.

**Peptidoglycan analysis**

The interpeptide bridge of the peptidoglycan structure of *Weisella cibaria* is different from the corresponding structure of *Weisella confusa*. The interpeptide bridge of *Weisella cibaria* contains serine, L-Lys–L-Ala–L-Ser–L-Ala (peptidoglycan type A3α), which is absent from the cell wall of *Weisella confusa*. Purified cell walls of *Weisella cibaria* strains LMG 17699<sup>T</sup> and LMG 18814 contain, besides muramic acid and glucosamine, the amino acids lysine, glutamic acid, serine and alanine in a molar ratio of 1:1:0.5:5.3:5, respectively. As reported before, unique peptidoglycan structures of *Weisella* species are of help in species identification (Collins et al., 1993), whereas carbohydrate fermentation profiles used alone for LAB identification may result in controversy (Milliere et al., 1989; Lyhs et al., 1999; Björkroth et al., 2000). Based on the API 50 CH Lactobacillus identification system (BioMérieux) all *Weisella cibaria* strains were identified as *Lactobacillus coprophilus*. Unfortunately, *Lactobacillus coprophilus* could not be included as a reference strain for this study. The type strain does not exist in any culture collection; therefore it is not a valid species. However, the interpeptide bridge structure in the peptidoglycan of *Lactobacillus coprophilus* (Plapp & Kandler, 1967) has been reported to be different from the structures of *Weisella cibaria* and *Weisella confusa*. Since the length and type of the interpeptide bridge is a strong species-specific phenotypic criterion (Schleifer & Kandler, 1972), this finding indicates separate species status between these three species.

**Numerical analysis of whole-cell protein and 16S and 23S rDNA RFLP patterns**

Numerical analysis of both whole-cell protein and 16S and 23S rDNA RFLP patterns resulted in species-specific clustering. Numerical analyses of whole-cell protein (Fig. 1) patterns, ClaI- (Fig. 2a) and, to some extent, HindIII- (Fig. 2c) based ribotypes were found to provide similar clustering with consistency in strain division between species-specific groups. In the EcoRI-based dendrogram (Fig. 2b) the *Weisella hellenica* type strain clustered together with two of the *Weisella cibaria* strains and *Weisella cibaria* LMG 18814 strains clustered together with *Weisella confusa* strains. The patterns generated by EcoRI digestion contained mainly a few large molecular mass fragments (Fig. 2b), subjecting the numerical analysis to errors due to the limited differences in the mobility of these fragments. Patterns like this do not provide a good matrix for numerical analysis which should be taken into account when selecting the enzymes. EcoRI ribotyping gave similar results also when *Leuconostoc* species were characterized (Björkroth et al., 2000) and its use cannot be recommended for species level identification of these organisms.

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**Fig. 1.** Numerical analysis of whole-cell protein patterns presented as a dendrogram.
**Fig. 2.** (a), (b) and (c) present Clal, EcoRI and HindIII ribopatterns, respectively, and the numerical analysis of the patterns is presented as a dendrogram. Left side of the banding patterns, high molecular masses, < 23 kbp in all patterns; right side of the banding patterns, low molecular masses, > 1000 bp in Clal and HindIII patterns and > 4000 bp in EcoRI patterns.

**Fig. 3.** Phylogenetic tree based on homologies of 1320 bp sequences in the 16S rDNA of Weissella type strains; Weissella cibaria is included.

### 16S rDNA sequence analysis

Fig. 3 shows the phylogenetic tree of genus Weissella type strains; Weissella cibaria is included, and is based on a comparison of a set of 1320 common nucleotide positions in the sequences analysed. According to the 16S rDNA sequence data, the Weissella confusa type strain possessed the highest similarity, 99.2%, to Weissella cibaria LMG 17699\(^T\) (unknown bases were excluded from the calculations). Only these two species were located in this branch of the phylogenetic tree. In our study, the degree of 16S rDNA sequence similarities ranged between 89.2 and 99.2% among the different Weissella species (data not shown). Some pairs of species, in particular Weissella confusa and Weissella cibaria, Weissella hellenica and Weissella paramesenteroides, and Weissella minor and Weissella viridescens, share 99.0% or more of their 16S rDNA sequences, excluding the comparison of (near) entire 16S rDNA sequences for setting a similarity cut-off value between the different species. Our study also shows that the topology of the trees based on protein pattern or ribopattern similarity differed from that of the phylogenetic tree, and therefore that the former methods should be used for species level identification and not phylogeny. This was also observed in a taxonomic study of Leuconostoc gasicomitatum and other Leuconostoc species (Björkroth et al., 2000).

### DNA–DNA hybridization results and DNA base composition

Table 2 presents the DNA–DNA hybridization results. The results show that Weissella cibaria strains shared DNA–DNA hybridization levels of 22–49% with the
Description of Weissella cibaria sp. nov.

Weissella cibaria (ci.ba’ri.a. L. adj. cibarius pertaining to food).

Cells are Gram-positive, short rods growing in pairs, 0.8–1.2 μm wide and 1.5–2.2 μm long. Non-motile. Peptidoglycan type is A3γ. t-Lys–t-Ala(t-Ser)–t-Ala. Colonies are small and greyish white and catalase-negative. Growth occurs at 15, 37 and 45 °C, but not at 4 °C. Heterofermentative, producing CO₂ from glucose. D- and L-lactic acid isomers are synthesized. Arginine is hydrolysed. Slime is produced from sucrose. None of the strains grew in the presence of 8% NaCl but all grew in 6.5% NaCl. The following carbohydrates were fermented: t-arabinose, D-xyllose, glucose, fructose, mannose, N-acetylglucosamine, amygdaline, arbutine, aesculin, salicine, cellobiose, maltose, saccharose and gentiobiose. Glycerol, erythritol, arabinose, ribose, L-xyllose, adonitol, β-methyl-D-xyloside, galactose, sorbose, rhamnose, dulcitol, inositol, mannotriol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, lactose, melibiose, trehalose, inulin, melezitose, raffinose, amygdalin, glycogen, xylitol, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate were not fermented. The DNA base ratio is 44–45 mol% G+C (thermal denaturation method). Weissella cibaria strains have been isolated from fermented food products and from clinical samples from humans and animals. The type strain is LMG 17699T which was isolated from chili bo in Malaysia. Its DNA base ratio is 44 mol% and its phenotypic characteristics are as described above. The EMBL accession no. of the 16S rRNA gene sequence of LMG 17699T is AJ295989. The type strain has been deposited in the BCCM/LMG and CCUG culture collections as LMG 17699T = CCUG 41967T; all other strains are available from the BCCM/LMG collection.

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