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# Novel PCR-based identification of *Weissella confusa* using an AFLP-derived marker

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ABSTRACT

An extensive use of *Weissella* (*W.*) *confusa* is currently being made for the production of a variety of fermented foods and beverages although some strains of this species have emerged as opportunistic pathogens for humans and animals. Nevertheless, no rapid methods are available for the reliable identification of *W. confusa.* We developed a novel PCR using AFLP (Amplified Fragment Length Polymorphism)-derived primers for the rapid and unequivocal identification of *W. confusa*. Fluorescent AFLP of 30 strains of *W. confusa*, *Leuconostoc citreum*, *Lactobacillus* (*Lb.*) *brevis*, *Lb. rossiae*, *Lb. plantarum* and *Lb. buchneri* allowed us to detect, purify and sequence several *W. confusa* specific AFLP fragments. The homology search in BLAST of a 303 bp nucleotide sequence revealed a ≤77% identity of the purified fragment with the *lep*A gene of several lactic acid bacteria. A PCR assay targeting 225 bp of this fragment was developed and tested against the DNA of 109 strains, including 34 foodborne and clinical *W. confusa* and 75 strains of 47 phylogenetically closely and distantly related species, resulting in 100% specificity with a detection limit of 16 pg. Being the first species-specific PCR to date developed for the rapid and unambiguous identification of *W. confusa*, this novel assay could be a reliable and efficient tool for detecting *W. confusa* not only in food and beverages, but also in clinical specimens, thus contributing to clarify its real significance in human and animal infections.



## 1. Introduction

The *Weissella* genus, first described by Collins et al. (1993), includes Gram-positive, obligate heterofermentative, catalase negative coccobacilli, producing D- or DL-isomers of lactic acid as main end-products of fermentation (Collins et al., 1993). To date this genus encompasses 13 validated species, including *Weissella* (*W.*) *kimchii* (Choi et al., 2002), which has been indicated by Ennahar and Cai (2004) as a later heterotypic synonym of *W. cibaria* (Björkroth et al., 2002). Bacteria of the *Weissella* genus have been isolated from a wide range of sources such as soil, fresh vegetables, meat, fish, fermented silage and fermented foods (Björkroth et al., 2002; Choi et al., 2002; Escalante-Minakata et al., 2008; Magnusson et al., 2002; Sirirat et al., 2008; Thapa et al., 2006; Valerio et al., 2009; Wang and Nishinno, 2008).

*W. confusa* is one of the most widespread *Weissella* species in food: strains of this species have been demonstrated to act as probiotic and technological. Indeed, Nam et al. (2002) reported the capability of a *W. confusa* strain to reduce the infectivity and persistence of *Helicobacter pylori*. Chun et al. (2007) isolated a *W. confusa* strain from human faeces, and tested it for its capability of converting isoflavone glucosides to aglycones in soymilk. A *W. confusa* strain was successfully employed in a multi-species semi-liquid ready-to-use sourdough starter (Gaggiano et al., 2007), whereas Di Cagno et al. (2009b) used strains of this species as autochthonous starters to ferment tomato juice as well as red and yellow peppers (Di Cagno et al., 2009a). Maina et al. (2008) reported a *W. confusa* strain as a suitable alternative to the widely used *Leuconostoc mesenteroides* B512F in the production of high amounts of linear dextran. A *W. confusa* strain useful for the efficient *in situ* production of dextrans and isomaltooligosaccharides in wheat sourdoughs without strong acidification was described by Katina et al. (2009). Given its widespread use for biotechnological purposes, rapid and reliable methods to unequivocally identify and detect *W. confusa* are therefore crucial to verify the correct advancement of the fermentation process, ascertaining the capability of the selected strains to dominate on the native microbial flora and achieving the traceability and the certification of the final products.

Despite the technological and/or probiotic role of *W. confusa*, strains of this species have been seldom indicated as causative agents of food spoilage (Diez et al., 2008; Morishita and Shiromizu, 1986; Santos et al., 2005). Other *W. confusa* strains have been isolated from human and animal clinical sources (Bantar et al., 1991; Björkroth et al., 2002; Green et al., 1990, 1991; Olano et al., 2001; Riebel and Washington, 1990). The polymicrobial nature of these cases did not allow to assign a certain clinical significance to this species. Lately, *W. confusa* has been recognised as the unique microbial agent of: i) a systemic infection in a Mona monkey (*Cercopithecus mona*) (Vela et al., 2003), ii) a fatal case of endocarditis (Flaherty et al., 2003), iii) a

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severe infective endocarditis of native valves (Shin et al., 2007), and iv) a postoperative osteomyelitis with chronic discharge in a young female (Kulwichit et al., 2008). These findings led to hypothesize *W. confusa* as an opportunistic human and animal pathogen and, once again, recall for a fast and accurate species identification to shed light on the clinical significance of this microorganism in infections and ensure the appropriate approach to patients if a severe infection is suspected.

The identification of *W. confusa* and the other *Weissella* species traditionally relies on their biochemical and physiological features such as the production of gas from carbohydrates, the presence within the cell wall of lysine and alanine joined with an intrapeptide bond, the hydrolysis of arginine, the formation of D,L-lactate and the ability to ferment different sugars (Björkroth et al., 2002; Collins et al., 1993; De Bruyne et al., 2008; Koort et al., 2006). Besides from being labour intensive and time consuming, these traditional methods as well as their advanced applications (such as cell wall composition analysis, whole-cell protein fingerprinting and fatty acid analysis) often lead to uncertain identification or even misidentification, especially in cases of phenotypically closely related species (Björkroth et al., 2002; De Graef et al., 2005; Koort et al., 2006; Kulwichit et al., 2008; Shin et al., 2007). 16S rDNA sequencing (Kulwichit et al., 2008; Valerio et al., 2009) and amplified ribosomal DNA restriction analysis (ARDRA) (Jang et al., 2002) have been used as alternative molecular approaches to identify and detect *W. confusa*. These methods require considerable time and skills and are not well suited for massive parallel testing as required in clinical and food microbial diagnostics, whereas species-specific polymerase chain reaction (PCR) assays may adequately meet these needs.

No PCR assays are to date available for the rapid and unambiguous detection of *W. confusa*, and no genome sequences of strains of this species have been deposited so far.

Informative polymorphic banding patterns can be obtained by amplified fragment length polymorphism (AFLP) allowing the differentiation with a high discriminatory power even of phylogenetically closely related bacteria without any prior information on their genomes (Janssen et al., 1996). This multi-locus fingerprinting technique combines the reliability of restriction fragment length polymorphism (RFLP) analysis with the flexibility and robustness of PCR using restriction site/adaptor-specific primers under stringent conditions (Vos et al., 1995). Semi-automated versions of this technique may be obtained by the fluorescent AFLP (fAFLP) using capillary array systems, fluorescently labelled primers and adequate analysis software (Zhao et al., 2000). However, AFLP is expensive and not all laboratories are equipped for performing it. To overcome these problems, AFLP markers may be converted into simple PCR markers to be used as diagnostic tools for the rapid, reliable, easy, and low cost detection of microorganisms.

In this study, we report the fAFLP typing of several lactic acid bacteria to detect, characterise and select specific AFLP markers allowing the development of a novel PCR-based assay for the rapid and unambiguous identification of *W. confusa*.

## 2. Materials and methods

### 2.1. Bacterial strains and growth medium

The bacterial strains used in this study are listed in Table 1. All wild strains were previously identified and characterised by conventional and/or molecular methods (Corsetti et al., 2001; Quero, 2006; Valerio et al., 2009).

The cultures were grown on the appropriate media (Oxoid, Basingstoke, UK), according to DSMZ (website: http://www.dsmz.de), BCCM/LMG (http://bccm.belspo.be/db/lmg_search_form.php) and ATCC (http://www.lgcstandards-atcc.org) catalogues before DNA extraction.

**Table 1**
Source, origin and results of the species-specific PCR of strains analysed in this study.

| Strain | Species | Source[a] | Origin | *W. confusa* species-specific PCR |
|---|---|---|---|---|
| 13F | *E. faecalis* | ISPA | Raw milk | – |
| 127GT0 | *E. faecium* | ISPA | Giuncata, fresh cheese | – |
| DSM20069$^T$ | *L. cremoris* | DSM | Unknown | – |
| DSM20684$^T$ | *L. garvieae* | DSM | Bovine mastitis | – |
| DSM20450$^T$ | *L. hordiniae* | DSM | Leafhopper (Hordnia circellata) | – |
| DSM4644 | *L. lactis* | DSM | Unknown | – |
| DSM20481$^T$ | *L. lactis* subsp. *lactis* | DSM | Unknown | – |
| DSM6634$^T$ | *L. piscium* | DSM | Diseased Rainbow yearling | – |
| DSM20686$^T$ | *L. plantarum* | DSM | Frozen peas | – |
| DSM20443$^T$ | *L. raffinolactis* | DSM | Raw milk | – |
| ATCC29643$^T$ | *L. alimentarius* | ATCC | Marinated fish product | – |
| ATCC14869$^T$ | *L. brevis* | ATCC | Human faeces | – |
| DPPMA33 | *L. brevis* | DPPMA | Unknown | – |
| 10A | *L. brevis* | ISPA | Sourdough | – |
| L5-21 | *L. brevis* | ISPA | Raw milk | – |
| LP5-6 | *L. brevis* | ISPA | Raw milk | – |
| P2 | *L. brevis* | ISPA | Sourdough | – |
| LB3 | *L. buchneri* | DPPMA | Unknown | – |
| ATCC27216 | *L. casei* subsp. *paracasei* | ATCC | Saliva of child | – |
| DSM20072$^T$ | *L. delbrvectii* subsp. *lactis* | DSM | Emmental cheese | – |
| ATCC29644$^T$ | *L. farciminis* | ATCC | Sausage | – |
| DSM20052$^T$ | *L. fermentum* | DSM | Fermented beets | – |
| ATCC8288$^T$ | *L. fructivorans* | ATCC | Unknown | – |
| LP17-1 | *L. johnsonii* | ISPA | Raw Milk | – |
| ATCC8041$^T$ | *L. pentosus* | ATCC | Unknown | – |
| ATCC10012 | *L. plantarum* | ATCC | Unknown | – |
| C21-41 | *L. plantarum* | ISPA | Durum wheat semolina | – |
| C25-3 | *L. plantarum* | ISPA | Durum wheat semolina | – |
| ATCC53103 | *L. rhamnosus* | ATCC | Human faeces | – |
| DPPMA71 | *L. rossiae* | DPPMA | Unknown | – |
| DPPMA72 | *L. rossiae* | DPPMA | Unknown | – |
| C21/11 | *L. rossiae* | ISPA | Durum wheat semolina | – |
| C21/47 | *L. rossiae* | ISPA | Durum wheat semolina | – |
| DPPMA70 | *L. rossiae* | DPPMA | Unknown | – |
| DPPMA73 | *L. rossiae* | DPPMA | Unknown | – |
| DSM8582 | *L. argentinum* | DSM | Raw milk | – |
| DSM5576$^T$ | *L. carnosum* | DSM | Vacuum-packaged beef | – |
| DSM5577$^T$ | *L. citreum* | DSM | Honey-dew of rye ear | – |
| C11/53 | *L. citreum* | ISPA | Mixture of durum wheat semolina | – |
| C11/54 | *L. citreum* | ISPA | Mixture of durum wheat semolina | – |
| C2/27 | *L. citreum* | ISPA | Durum wheat semolina | – |
| C2/28 | *L. citreum* | ISPA | Durum wheat semolina | – |
| C2/31 | *L. citreum* | ISPA | Durum wheat semolina | – |
| DSM20189$^T$ | *L. fallax* | DSM | Sauerkraut | – |
| DSM20349$^T$ | *L. fructosum* | DSM | Flowers | – |
| DSM20346$^T$ | *L. mesenteroides* subsp. *cremoris* | DSM | Hansen's dried starter powder | – |
| DSM20343$^T$ | *L. mesenteroides* subsp. *mesenteroides* | DSM | Fermenting olives | – |
| ATCC9135 | *L. mesenteroides* | ATCC | Unknown | – |
| DSM20193$^T$ | *L. pseudomesenteroides* | DSM | Cane juice | – |
| DSM20597$^T$ | *M. caseolyticus* | DSM | Dairy products (cow's milk) | – |
| DSM20231$^T$ | *S. aureus* | DSM | Human pleural fluid | – |

**Table 1** (*continued*)

| Strain | Species | Source[a] | Origin | *W. confusa* species-specific PCR |
|---|---|---|---|---|
| DSM20266$^T$ | *S. xylosus* | DSM | Human skin | − |
| DSM15879$^T$ | *S. macedonicus* | DSM | Greek Kasseri cheese | − |
| DSM20617$^T$ | *S. thermophilus* | DSM | Pasteurized milk | − |
| DSM15878$^T$ | *W. cibaria* | DSM | Chili bo | − |
| C22-2 | *W. cibaria* | ISPA | Durum wheat semolina | − |
| C21-43 | *W. cibaria* | ISPA | Durum wheat semolina | − |
| C3-3 | *W. cibaria* | ISPA | Durum wheat semolina | − |
| C3-4 | *W. cibaria* | ISPA | Durum wheat semolina | − |
| C43-M1 | *W. cibaria* | ISPA | Whole durum wheat semolina | − |
| C3-19 | *W. cibaria* | ISPA | Durum wheat semolina | − |
| C4-21 | *W. cibaria* | ISPA | Durum wheat semolina | − |
| C2-23 | *W. cibaria* | ISPA | Durum wheat semolina | − |
| C2-33 | *W. cibaria* | ISPA | Durum wheat semolina | − |
| LMG11983 | *W. confusa* | LMG | Grass silage | + |
| LMG14040 | *W. confusa* | LMG | Dog ear (otitis), Belgium | + |
| LMG16883 | *W. confusa* | LMG | Human drain, Sweden | + |
| LMG17670 | *W. confusa* | LMG | Human blood, Sweden | + |
| LMG17671 | *W. confusa* | LMG | Human gall, Sweden | + |
| LMG17695 | *W. confusa* | LMG | Chili bo, Malaysia | + |
| LMG17696 | *W. confusa* | LMG | Chili bo, Malaysia | + |
| LMG17698 | *W. confusa* | LMG | Chili bo, Malaysia | + |
| LMG17705 | *W. confusa* | LMG | Chili bo, Malaysia | + |
| LMG17709 | *W. confusa* | LMG | Chili bo, Malaysia | + |
| LMG17718 | *W. confusa* | LMG | Chili bo, Malaysia | + |
| LMG18475 | *W. confusa* | LMG | Tapai, Malaysia | + |
| LMG18476 | *W. confusa* | LMG | Tapai, Malaysia | + |
| LMG18477 | *W. confusa* | LMG | Tempeh, Malaysia | + |
| LMG18478 | *W. confusa* | LMG | Tapai, Malaysia | + |
| LMG18479 | *W. confusa* | LMG | Tapai, Malaysia | + |
| LMG18480 | *W. confusa* | LMG | Tapai, Malaysia | + |
| LMG18500 | *W. confusa* | LMG | Dog (autopsy), Sweden | + |
| LMG18503 | *W. confusa* | LMG | Human faeces, Sweden | + |
| LMG18505 | *W. confusa* | LMG | Human faeces, Sweden | + |
| LMG18815 | *W. confusa* | LMG | Dialysis patient faeces, Belgium | + |
| LMG18816 | *W. confusa* | LMG | Dialysis patient faeces, Belgium | + |
| DSM20196$^T$ | *W. confusa* | DSM | Sugar cane | + |
| DSM20194 | *W. confusa* | DSM | Soured carrot mash | + |
| C6-1 | *W. confusa* | ISPA | Durum wheat semolina | + |
| C5-2 | *W. confusa* | ISPA | Durum wheat semolina | + |
| C2-13 | *W. confusa* | ISPA | Durum wheat semolina | + |
| C3-17 | *W. confusa* | ISPA | Durum wheat semolina | + |
| C2-17 | *W. confusa* | ISPA | Durum wheat semolina | + |
| C4-17 | *W. confusa* | ISPA | Durum wheat semolina | + |
| C5-1 | *W. confusa* | ISPA | Durum wheat semolina | + |
| C6-8 | *W. confusa* | ISPA | Durum wheat semolina | + |
| C45-M1 | *W. confusa* | ISPA | Whole durum wheat semolina | + |
| C3-9 | *W. confusa* | ISPA | Durum wheat semolina | + |
| DSM19935$^T$ | *W. ghanensis* | DSM | Fermented cocoa beans | − |
| DSM20190$^T$ | *W. halotolerans* | DSM | Sausage | − |
| DSM7378$^T$ | *W. hellenica* | DSM | Sausage, naturally fermented | − |
| DSM20593$^T$ | *W. kandlerii* | DSM | Desert spring | − |
| LMG17699$^T$ | *W. kimchii* | LMG | Chili bo | − |
| DSM15830$^T$ | *W. koreensis* | DSM | Kimchi | − |
| DSM20014$^T$ | *W. minor* | DSM | Milking machine slime | − |
| DSM20288$^T$ | *W. paramesenteroides* | DSM | Fermented sausages, dry salami | − |
| DSM14420$^T$ | *W. soli* | DSM | Garden soil | − |
| DSM15832$^T$ | *W. thailandensis* | DSM | Fermented fish | − |
| ATCC12706$^T$ | *W. viridescens* | DSM | Cured meat products | − |

[a] ATCC, American Type Culture Collection, Rockville, Maryland, USA; DPPMA, Dipartimento Protezione Piante e Microbiologia Applicata, University of Bari, Italy (DPPMA strains were kindly provided by Dr. Di Cagno); DSM, Deutsche Sammlung von Mikroorganism, Braunschweig, Germany; ISPA, Institute of Sciences of Food Production, National Research Council, Bari, Italy; LMG/BCCM, Belgian Coordinated Collections of Microorganisms, Laboratorium Microbiologie Gent Culture Collection, Universiteit Gent, Belgium.

### 2.2. DNA isolation

The isolation of DNA was carried out from one millilitre of an overnight broth-culture by the DNeasy Blood and Tissue kit (Qiagen, Milan, Italy), following the manufacturer's instructions. The purity and quantification of DNA were assessed spectrophotometrically (Nanodrop® ND-1000, Nanodrop Technologies, Inc., Wilmington, DE, USA) and by agarose gel electrophoresis (Sambrook et al., 1989) with known amounts of lambda DNA marker II (Roche S.p.a., Milan, Italy) as standard.

### 2.3. fAFLP

Thirty strains belonging to six lactic acid bacteria species (Fig. 1), i.e. *Lb. plantarum*, *Lb. rossiae*, *W. confusa*, *Lb. brevis*, *Lb. buchneri*, and *Leuc. citreum* were used for fAFLP analysis. The AFLP Microbial Fingerprinting kit (Applied Biosystems, Foster City, CA, USA) was used following the manufacturer's instructions.

To establish which selective primer pairs yielded the highest quality of AFLP fingerprints, various combinations of *Eco*RI and *Mse*I adaptor-specific primers (Applied Biosystems) with base selection (*Eco*RI-0/*Mse*I-0, *Eco*RI-G/*Mse*I-0, *Eco*RI-A/*Mse*I-T, *Eco*RI-C/*Mse*I-G) were used. The *Eco*RI primers were provided labelled with fluorescent dye (Applied Biosystems).

Preselective and selective PCR amplifications were carried out following the AFLP microbial fingerprinting protocol of Applied Biosystems in a Thermal Cycler 9700 (Applied Biosystems). One microlitre of each selective amplification product was mixed with 20 μL of formamide and 1 μL of GeneScan — 500 (ROX) size standard (Applied Biosystems), ranging from 35 to 500 bp in size. Further denaturation of each mixture was achieved by heating for 3 min at 95 °C, followed by a rapid cooling on ice. Samples were then processed for capillary electrophoresis using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Fragments containing an adapter specific for the restriction site created by the *Eco*RI restriction enzyme were visualised due to the 5′ — end labelling of the corresponding primer with the fluorescent dye. The resulting electrophoretic patterns were tracked and normalised using the GeneScan Software (Applied

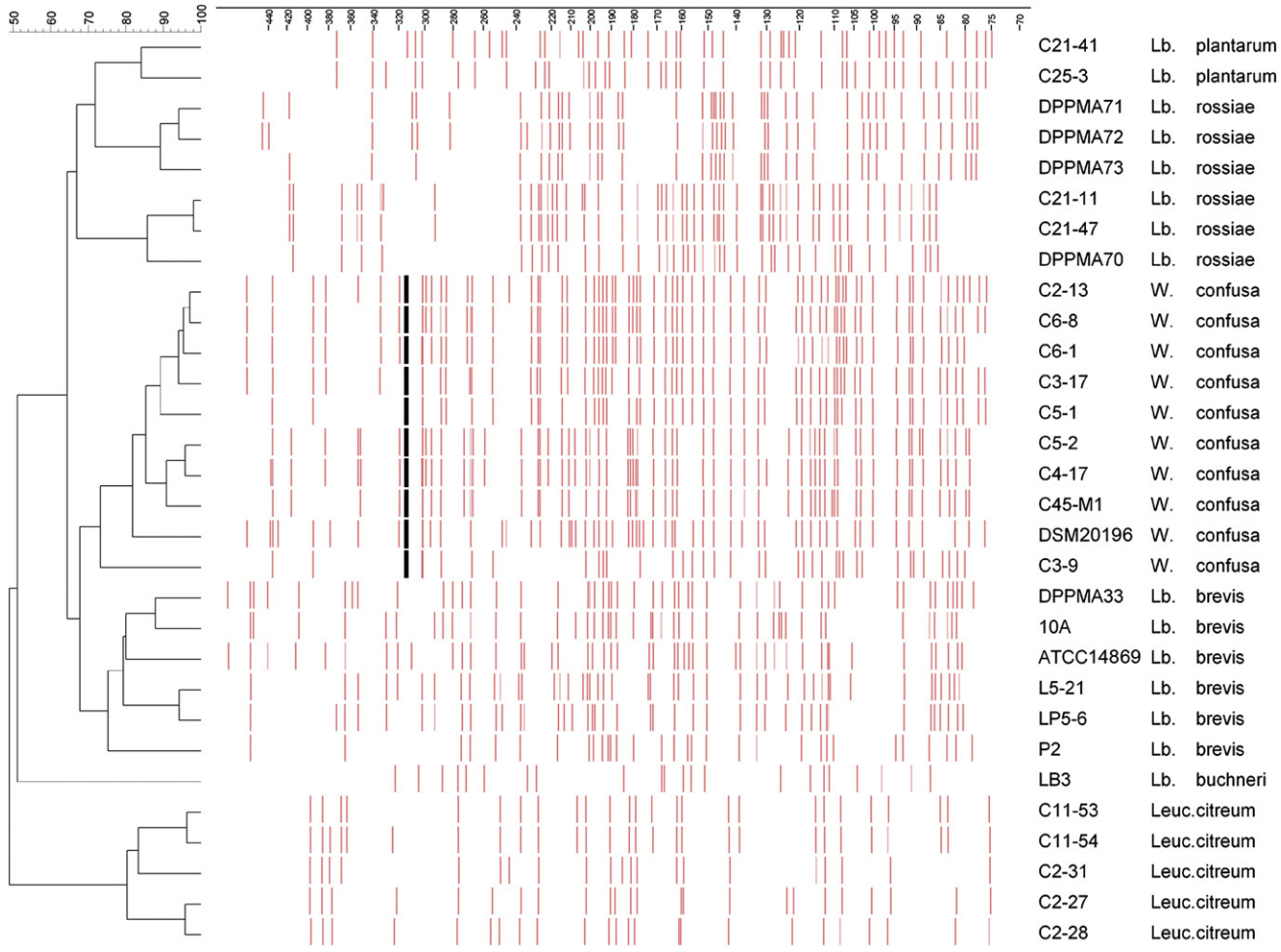


**Fig. 1.** Dendrogram and banding patterns of 30 lactic acid bacteria strains analysed by AFLP typing. The dendrogram was constructed with the UPGMA method after calculation of the band pattern similarity (%) using the Dice's coefficient. The numbers above the banding pattern indicate the fragment size in bp. The *W. confusa* specific AFLP marker used for the species-specific PCR assay developed in this study is highlighted in bold black.

Biosystems). All electropherograms were visually inspected and compared for polymorphic peaks with the same software. Peak height threshold was set at 70 fluorescent arbitrary units so that any peak below this value was excluded from the analysis. Normalised tables of peaks, containing fragments ranging from 70 to 500 bp, were transferred to the Bionumerics 5.1 software (Applied Maths, Sint-Martens-Latem, Belgium) for numerical analysis. Fingerprint similarity values were calculated using the Dice's coefficient and the dendrogram was constructed using the UPGMA (unweighted pair group method with arithmetic means) algorithm.

### 2.4. Isolation of AFLP fragments

To obtain the AFLP fragments corresponding to the specific fAFLP peaks selected, the following steps were carried out: i) AFLP fragments obtained by using a specific selective primer pair were separated by electrophoresis on a 6% polyacrylamide gel (16×16 cm) using a Bio-Rad D-Code apparatus (Bio-Rad Laboratories Richmond CA, USA) at 50 V for 10 min and 80 V for 8 h in 1X TBE (Tris-Borate-EDTA) buffer; ii) presumptive species-specific bands were purified in water according to Ampe et al. (1999); iii) one microlitre of the eluted DNA of each AFLP band was re-amplified by using selective primers having equal or different numbers and/or types of nucleotide extensions; iv) once the purity of the fragment was ascertained (via fAFLP), it was sequenced by using both unlabeled forward and reverse primer.

### 2.5. Design of the W. confusa species-specific PCR assay

Based on the nucleotide sequences of the AFLP-derived marker, primers were designed using the Primer Express 2.0 software (Applied Biosystems). Candidate primers sequences were examined for specificity following BLAST (www.ncbi.nlm.nih.gov/blast/) and EMBL nucleotide sequence (www.ebi.ac.uk/embl/) database searches for DNA sequences. BP40F (5′-GGCGGATTGGTCTCTTTTTG-3′) and BP264R (5′-CACGCTCAGTAACCGTGTGC-3′) resulted to be the best primer set and therefore it was selected for the *W. confusa* species-specific PCR.

Each reaction amplification mixture (25 μL final volume) contained 10 ng of genomic DNA, forward and reverse primer at a concentration of 300 mM, 0.2 μM of each deoxynucleoside triphosphate, and 0.3 μL of *Taq* DNA polymerase (5 U/ μL) (Sigma, Milan, Italy) in the supplied buffer. Polymerase chain reactions were carried out in a 9700 Thermal Cycler (Applied Biosystems) by using the following optimised two step amplification protocol: 5 min at 95 °C, 35 cycles at 95 °C for 15 s and 60 °C for 45 min. Amplification products were checked by agarose gel electrophoresis (2% w/v) in 1X TBE buffer, stained with 0.5 μg $mL^{-1}$ of ethidium bromide (Sigma) and visualised by UV, using the Chemidoc apparatus (Bio-Rad).

### 2.6. PCR sensitivity and specificity

To determine the detection limit of the *Weissella confusa* species-specific PCR assay herein developed, different amounts (from 50 to

10 ng), and five fold dilutions (from 10 ng to 0.64 pg) of purified genomic DNA from *W. confusa* DSM20196$^T$ were tested in triplicate.

The specificity of our assay was evaluated in terms of inclusivity and exclusivity, according to the Microval Protocol of the ISO 16140:2003 (Anonymous, 2003). Inclusivity is intended as the ability of the PCR method to detect the target analyte from a wide range of strains whereas exclusivity is the lack of response from a relevant range of closely related, non target strains (Hoorfar and Cook, 2002). The inclusivity and exclusivity of the assay were assessed *in silico*, by homology searches of our primers in the nucleotide databases, as above reported, and then *in vitro*. In the last case, 10 ng of the bacterial DNA template from 109 strains (Table 1), chosen based on their phylogenetic relationship to *W. confusa* (Björkroth et al., 2002; Chelo et al., 2007; Nigatu, 2000), were tested in triplicate. The specificity of our assay was also evaluated against a DNA mixture of the type strains (2 ng each) belonging to all the *Weissella* species to date known and other closely related lactic acid bacteria (LAB).

### 2.7. Nucleotide sequencing and sequence analysis

The PCR products were purified with the QIAquick gel extraction kit (Qiagen), and spectrophotometrically quantified (with Nanodrop ND-1000). Thereafter, purified amplicons were used for sequencing with the ABI PRISM BigDye Terminator Ready Reaction kit V 3.1 (Applied Biosystems), using either primers BP40F/264R or both unlabeled AFLP selective forward and reverse primers, and run on an Applied Biosystems ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

The sequences were analyzed by the Gene Scan software (Applied Biosystems), and aligned via the Bionumerics version 5.1 (Applied Maths). A search for DNA similarity was performed with the GenBank (Altschul et al., 1997) and EMBL (Stoesser et al., 1997) databases.

## 3. Results and discussion

Among the various selective primer pairs assayed, the *Eco*RI-A/*Mse*I-T primer pair was considered the most effective, yielding the highest quality AFLP fingerprints. In particular, a high number of peaks which were also too close to each other were obtained by using either the *Eco*RI-0/*Mse*I-0 or the *Eco*RI-G/*Mse*I-0 primer pair, resulting in peak patterns which were too complicated to analyse. The addition of selective nucleotides to the core primers led to a reduction in the number of peaks obtained via selective amplification. However, the *Eco*RI-C/*Mse*I-G primer pair showed a lower discriminatory power than the *Eco*RI-A/*Mse*I-T primer pair in terms of amount of polymorphic peaks allowing the further isolation of the relative amplicons (data not shown).

Peak profiles produced with the *Eco*RI-A/*Mse*I-T primer set were analysed by the Bionumerics software and visualised as banding patterns. Informative polymorphic banding patterns were obtained for all strains analysed, achieving a typeability of 100%. Banding patterns of *Lb. buchneri*, *Leuc. citreum*, *Lb. plantarum*, *Lb. brevis*, *Lb. rossiae*, and *W. confusa* contained on average 22, 24, 44, 45, 46 and 61 discrete bands, respectively, ranging in size from 75 to 486 bp (Fig. 1). Clear polymorphism both within and between species were obtained by AFLP analysis. The strains clustered into six distinct AFLP groups, one per each different lactic acid bacteria species analysed (Fig. 1).

fAFLP analysis with primer combination *Eco*RI-A/*Mse*I-T made it feasible to identify more than one potential species-specific peak (Fig. 1). AFLP fragments appearing to be unique to *W. confusa* were isolated, purified, sequenced, and searched against GenBank and EMBL, as reported in the materials and methods section. In order to characterise the most promising AFLP peak, the relevant fragment was isolated in the best conditions of purity: selective amplification of the *W. confusa* C5-2 pre-selective amplicon was performed by using the four possible combinations of *Mse*I-T primer with *Eco*RI-AT, *Eco*RI-AG, *Eco*RI-AC, or *Eco*RI-AA selective primers. Only the amplification product obtained by using the selective primer set *Eco*RI-AA/*Mse*T exhibited the same mobility as that of the selected AFLP marker.

Consequently, the remaining *Eco*RI-AA/*Mse*I-T amplified product was separated by polyacrylamide gel electrophoresis (PGE); the band of interest was carefully eluted from the gel, purified, re-amplified, confirmed via fAFLP and PGE, and, finally, sequenced by using both unlabeled forward and reverse primer.

The BLAST homology search on the public nucleotide database of the resulting 303 bp nucleotide sequence (GenBank accession number: GQ438819) revealed a discrete nucleotide sequence identity ($\leq$77%) of the eluted DNA fragment to the *lep*A gene of LAB species belonging to genera other than *Weissella* as well as of non LAB species. The best hit was the *lep*A gene of *Leuc. mesenteroides* subsp. *mesenteroides* ATCC 8293 (GenBank accession number: CP000414; nucleotides 1812–1677): 77% identity, Blast e-value = 2 $e^{-21}$. The *lep*A is a chromosomally located gene, present at least as one copy in almost every bacterial genome (Evans et al., 2008), encoding the GTP-binding protein LepA. This protein, also known as Elongation Factor 4 (EF4), localized in the cytoplasmic membrane, promotes the back-translocation of mistranslocated ribosomes, improving the fidelity of translation (Qin et al., 2006). The lack of complete genome sequences of strains belonging to the *Weissella* genus as well as the fact that this genus owes its genus status to the re-classification of certain species of *Leuconostoc* and heterofermentative lactobacilli (Collins et al., 1993) clearly motivates the results of the homology search. Moreover, the high variability among the *lep*A genes available in the BLAST and EMBL nucleotide sequence databases also leads to hypothesize that this gene might be useful for phylogenetic studies as well as for the discrimination of all *Weissella* species. Ongoing work in our laboratories is focusing on characterising the flanking regions of the AFLP-derived fragment in order to obtain the full sequence of the *lep*A gene of *W. confusa* and of the other *Weissella* species and explore the possibility to use it as a phylogenetic marker or as a *Weissella* species identification tool.

Based on the nucleotide sequence of the 303 bp AFLP-derived marker (GenBank accession number: GQ438819), the set of primers BP40F (position 40–59)/BP264R (position 264–245) was designed to amplify, by PCR, a 225 bp fragment specifically in *W. confusa.* The homology searches of our primers in the nucleotide databases revealed no 100% identical sequences other than that of the AFLP-derived fragment we deposited so far (GenBank accession number: GQ438819), confirming "*in silico*" the specificity of our assay. The PCR was then optimised for sensitivity and specificity by titrating either the concentration of primers or deoxynucleoside triphosphates, or the amount of Taq polymerase (data not shown).

The sensitivity of the optimised PCR assay was evaluated by testing different amounts of purified genomic DNA from *W. confusa* DSM20196$^T$. As reported in Fig. 2, as few as 16 pg of target DNA could be detected, with increasing band intensity from 16 pg to 10 ng of target DNA (plateau). By using the optimised PCR conditions, a single product of the expected size was obtained only when the DNA from *W. confusa* was present in the reaction mixture (Fig. 2). Our PCR assay was evaluated against the DNA of 109 strains, including: i) 34 *W. confusa* recovered from clinical specimens and food samples, ii) 10 *W. cibaria*, iii) 11 reference strains belonging to the other *Weissella* species to date known, and iv) 54 strains representing 35 phylogenetically closely (namely *Leuconostoc*, oenococci, and lactobacilli) and distantly (the *Macrococcus caseolyticus* type strain, streptococci, lactococci and staphylococci) related bacterial species and subspecies (Table 1). As reported in Table 1, the primers we designed showed very high specificity for the detection of *W. confusa* regardless of the origin and intra-species variability of the considerable number of strains analysed, resulting in 100% inclusivity and exclusivity.

Discriminating *W. confusa* from the other *Weissella* species is a hurdle, since they are phylogenetically and phenotypically closely

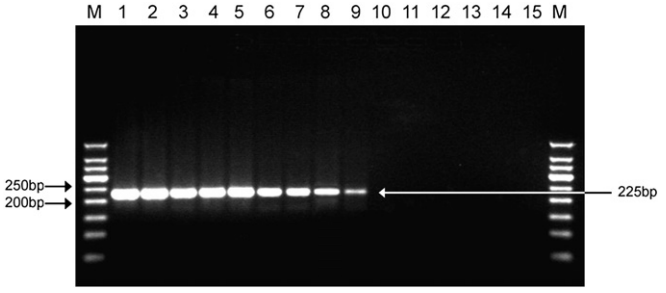


**Fig. 2.** Sensitivity and species-specificity of the PCR assay for *W. confusa*. Lanes 1–11: 50 ng, 40 ng, 30 ng, 20 ng, 10 ng, 2 ng, 400 pg, 80 pg, 16 pg, 3.2 pg and 0.64 pg of *W. confusa* DSM20196$^T$ genomic DNA. Lanes 12–14: 10 ng of genomic DNA of *W. cibaria* DSM15878$^T$, *Leuc. pseudomesenteroides* DSM20193$^T$ and *Lb. fermentum* DSM20052$^T$. Lane 15: deionised sterile water. Lanes M: GelPilot 50 bp Ladder DNA Marker (Qiagen).

related. In particular, *W. confusa* is distinguishable from its closest relative *W. cibaria* due to the capability of the former to ferment galactose and xylose, while the latter could produce acid only from L-arabinose (Björkroth et al., 2002). Nonetheless, due to both the subjectivity in the interpretation of results and the variability and instability of the phenotypic characters, relying solely upon these criteria may lead to misidentifications (De Graef et al., 2005) which may have dangerous consequences especially in the case of *W. confusa* as opportunistic pathogen (Kulwichit et al., 2008; Shin et al., 2007). Given the high similarity found between *W. confusa* and *W. cibaria* 16S rRNA gene (99.2%; Björkroth et al., 2002), misidentifications of these species may occur due to non complete sequencing of this gene. Moreover, the genus-specific 16S-amplified ribosomal DNA restriction analysis developed by Jang et al. (2002) to allow the identification of *Weissella* species could be ineffective for *W. confusa* since this method was not proven for *W. cibaria* and the other species successively added to this genus.

The robustness and reliability of our PCR assay is therefore strengthened by the absence of cross reaction with all the control strains, including the closest *W. confusa*'s relative *W. cibaria* (Table 1). Further evidence of the specificity of our PCR assay was obtained by the BLAST analysis (100% identity) of the *W. confusa* C5-2 amplicon (GenBank accession number: GQ438819) after its purification, reamplification and sequencing (data not shown).

Finally, to test the robustness and efficacy of the method even in presence of background microflora, we evaluated the ability to detect *W. confusa* in mixtures of different bacterial genomic DNAs. For this purpose, the species-specific PCR assay was performed using a DNA mixture of all the *Weissella* species to date known and some other LAB species, in the presence or absence of genomic DNA from *W. confusa* DSM20196$^T$. As shown in Fig. 3, the amplicon of the expected size was obtained only when *W. confusa* DNA was present in the PCR mixture, indicating that the presence of competing DNA, even of strictly related species, had no inhibitory effect on our PCR assay, making it a reliable tool for the detection of *W. confusa* from mixed cultures. Ongoing work in our laboratories is aimed at transferring this assay to a real time PCR platform and ascertaining its effectiveness in direct monitoring of *W. confusa* in food, beverages, and clinical specimens.

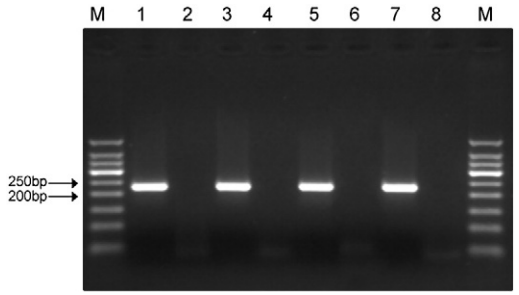


**Fig. 3.** Effectiveness of the species-specific PCR assay against DNA mixtures from different lactic acid bacteria (2 ng of DNA per each species) in presence and absence of *W. confusa* DSM20269$^T$ genomic DNA. Lanes 1 and 2: *W. cibaria* DSM15878$^T$, *W. ghanensis* DSM19935$^T$, *W. halotolerans* DSM20190$^T$, *W. hellenica* DSM7378$^T$. Lanes 3 and 4: *W. kandlerii* DSM20593$^T$, *W. kimchii* LMG17699$^T$, *W. koreensis* DSM15830$^T$, *W. minor* DSM20014$^T$. Lane 5 and 6: *W. paramesenteroides* DSM20288$^T$, *W. soli* DSM14420$^T$, *W. thailandensis* DSM15832$^T$, *W. viridescens* ATCC12706$^T$. Lane 7 and 8: *Lb. fermentum* DSM20052$^T$, *Lb. fructivorans* ATCC8288$^T$, *Lb. brevis* ATCC14869$^T$, *Lb. alimentarius* ATCC29643$^T$. In lanes 1, 3, 5, and 7 *W. confusa* DSM20196$^T$ genomic DNA (10 ng) was also present in the mixture. Lanes M: GelPilot 50 bp Ladder DNA Marker (Qiagen).

In conclusion, the protocol of conversion of AFLP markers into PCR markers herein used allowed us to develop a PCR assay highly specific and reliable for the rapid and unambiguous detection of *W. confusa*. As far as we know, this is the first species-specific PCR assay for *W. confusa*. This molecular approach, overcoming the limitations of the traditional identification procedures, will undoubtedly be a useful and reliable tool not only in the field of the applied food microbiology, allowing the monitoring of *W. confusa* in food and beverages, but also in clinical microbiology, permitting the early and accurate detection of *W. confusa* in clinical specimens, thus contributing to ascertain the actual clinical significance of this microorganism in human and animal infections.

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