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# AFLP protocol comparison for microbial diversity fingerprinting

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## Abstract

Over the last decade, several methods based on genomic DNA have been developed for the identification and genotyping of prokaryotic and eukaryotic organisms. These genomic methods differ regarding taxonomic range, discriminatory power, reproducibility, and ease of interpretation and standardization. The amplified fragment length polymorphism (AFLP) technique is a very powerful DNA fingerprinting technique for DNA of any source or complexity, varying in both size and base composition. In addition, this method shows high discriminatory power and good reproducibility allowing it to be efficient in discriminating at both the species and strain levels. The development and application of AFLP have allowed significant progress in the study of biodiversity and taxonomy of microorganisms. In the last years, the Applied Biosystems AFLP Microbial Fingerprinting Kit, now out of production, was widely used in various studies to perform AFLP characterization of selected bacteria strains (described by Vos et al. (Nucleic Acids Res 23(21):4407–4414, 1995)). Its replacement gives the possibility for laboratories to continue the use of the previous AFLP data as a reference for bacteria genetic fingerprinting analysis in biodiversity studies. To overcome this issue a result comparison, by using an improved AFLP protocol and the AFLP commercial kit, was performed. In particular, previous results on different species (*Listeria monocytogenes*, *Lactobacillus plantarum*, and *Streptococcus thermophilus*) obtained with the commercial kit were compared with the improved AFLP procedure to validate the protocol. When compared with the AFLP Microbial Fingerprinting Kit, the improved protocol shows high reproducibility, resolution, and overall, is a faster method with lower costs.

**Keywords** AFLP protocol · Bacterial diversity · Genome polymorphisms · Phylogenetic analysis

## Introduction

In the recent decades, several PCR-based fingerprinting methods have been developed and improved for bacteria genomic fingerprinting and genome screening purposes. The amplified ribosomal DNA restriction analysis (ARDRA) (Vaneechoutte et al. 1992; Gulitz et al. 2013), the automated ribosomal intergenic spacer analysis (ARISA) (Kovacs et al. 2010), the randomly amplified polymorphic DNA (RAPD) (Cocconcelli et al. 1995; Perin et al. 2017), length heterogeneity-PCR (LH-PCR) (Lazzi et al. 2004; Savo Sardaro et al. 2018), the amplified fragment length polymorphism (AFLP) (Zabeau and Vos 1993; Vos et al. 1995; Janssen et al. 1996), and the high-throughput metagenomics (De Filippis et al. 2017; Garofalo et al. 2017) are all useful tools in studying microbial communities. Among them, the AFLP technique has been largely used for genomic fingerprinting of DNA from a variety of sources. As widely reported in the literature, AFLP is a valuable technique for the classification of bacteria at the species and strain levels with high discriminatory power and good reproducibility (Janssen et al. 1996; Blears et al. 1998; Savelkoul et al. 1999; Jarraud et al. 2002).

AFLP fingerprinting provides several advantages over other techniques (Curtin et al. 2007). Firstly, prior knowledge of a microorganism's genome sequence is not necessary. Moreover, AFLP alleles can be fluorescently labeled, allowing a parallel characterization of several samples in automatic genome analyzers. Once the technique is fine-tuned, it

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is possible to obtain accurate information rapidly to allow for efficient identification and differentiation of species and strains. The development and application of AFLP as a fingerprinting method has led to significant progress in the study of genetic diversity and taxonomy of bacteria (Heir et al. 2000; Giraffa et al. 2001; Cocolin and Ercolini 2008; Cappello et al. 2008; Lazzi et al. 2009; Di Cagno et al. 2010; Lévesque et al. 2012; Nabhan et al. 2012; Hamza et al. 2012; Bernini et al. 2013; Jérôme et al. 2016). Most of the bacteria strain characterizations, in the past years, was developed following the Applied Biosystems AFLP Microbial Fingerprinting Kit protocol, according to the manufacturer's instructions. This paper reports the comparison between an improved AFLP protocol and the commercial AFLP microbial kit, taking into consideration the increasing allele amplification efficiency and resolution as well as its time-saving and cost-effective features.

## Materials and methods

### Bacteria and growth conditions

Twenty-one strains isolated from different food matrices were used in this study (Table 1). They include seven *Streptococcus thermophilus*, seven *Listeria monocytogenes*, and seven *Lactobacillus plantarum* strains. Bacterial strains were maintained as frozen stocks (− 80 °C) in M17 (*S. thermophilus*), TSB broth (*L. monocytogenes*), and MRS (*L. plantarum*) (Oxoid, Milan, Italy) supplemented with 15% glycerol (*w*/*v*). Before use, the cultures were propagated twice with a 3% (*v*/*v*) inoculum into the appropriate media and incubated at 42 °C (*S. thermophilus*), 37 °C (*L. monocytogenes*), and 30 °C (*L. plantarum*) for 24 h in optimal growth conditions. All *S. thermophilus* and *L. monocytogenes* strains belong to the collection of Food and Drug Department, University of Parma, Italy; seven *L. plantarum* strains (POM1, POM31, POM43, POM40, POM8, C6, POM38) were kindly given by the Department of Soil, Plant, and Food Science, University of Bari, Italy.

**Table 1** Strains used in this study

| Species | Strains | Source |
|---|---|---|
| *S. thermophilus* | 100 | Pecorino Toscano cheese |
| *S. thermophilus* | 145 | Pecorino Toscano cheese |
| *S. thermophilus* | 159 | Pecorino Toscano cheese |
| *S. thermophilus* | 418 | Pecorino Toscano cheese |
| *S. thermophilus* | 4027 | Pecorino Toscano cheese |
| *S. thermophilus* | 4028 | Pecorino Toscano cheese |
| *S. thermophilus* | 4042 | Pecorino Toscano cheese |
| *L. plantarum* | POM1 | Tomato |
| *L. plantarum* | POM8 | Tomato |
| *L. plantarum* | POM31 | Tomato |
| *L. plantarum* | POM38 | Tomato |
| *L. plantarum* | POM40 | Tomato |
| *L. plantarum* | POM43 | Tomato |
| *L. plantarum* | C6 | Carrot |
| *L. monocytogenes* | Lm6 | Gorgonzola cheese |
| *L. monocytogenes* | Lm9 | Gorgonzola cheese |
| *L. monocytogenes* | Lm34 | Gorgonzola cheese |
| *L. monocytogenes* | Lm35 | Gorgonzola cheese |
| *L. monocytogenes* | Lm40 | Gorgonzola cheese |
| *L. monocytogenes* | Lm41 | Gorgonzola cheese |
| *L. monocytogenes* | Lm44 | Gorgonzola cheese |

### AFLP analysis

#### Preparation of primary template for AFLP analysis

The AFLP procedure was performed according to the method of Vos et al. (1995) with the modifications described below. Restriction-ligation reactions were performed in a final volume of 50 μl containing 5 μl of 1× T4 DNA ligase buffer with 1 μl of 1 mM ATP (New England Biolabs, Ipswich, MA, USA), 10 μl of 250 ng/μl BSA (New England Biolabs, Ipswich, MA, USA), 1 μl of 10 mM ATP (Invitrogen S.R.L., Milano, Italy), 0.25 μl of 20 U/μl of *Eco*RI (New England Biolabs, Ipswich, MA, USA), 0.5 μl of 10 U/μl of *Mse*I (New England Biolabs, Ipswich, MA, USA), and 500 ng of genomic DNA. Two different adapters (BMR Genomics, Padova, Italy) (sequences shown in Table 2), one for the *Eco*RI sticky ends and one for the *Mse*I sticky ends, were ligated to the DNA by adding to the reaction of a mix containing 1 μl of 5 pmol/μl of *Eco*RI adaptor, 1 μl of 50 pmol/μl of *Mse*I adaptor, and 0.1 μl of 200 U/μl of T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). The reaction was incubated for 4 h at 37 °C. Two replications for each sample were performed. The digested-ligated DNA product to be used as templates for the first amplification reaction was diluted 10-fold with RNAse- and DNAse-free water and stored at − 20 °C.

**Table 2** Primers used for AFLP analysis

| Primer name | Sequence (5′-3′) |
|---|---|
| *Eco*RI-0 | GACTGCGTACCAATTC (labeled FAM 5′) |
| *Mse*I-0 | GATGAGTCCTGAGTAA |
| *Eco*RI-A | GACTGCGTACCAATTCA (labeled FAM 5′) |
| *Mse*I-A | GATGAGTCCTGAGTAAA |
| *Mse*I-C | GATGAGTCCTGAGTAAC |

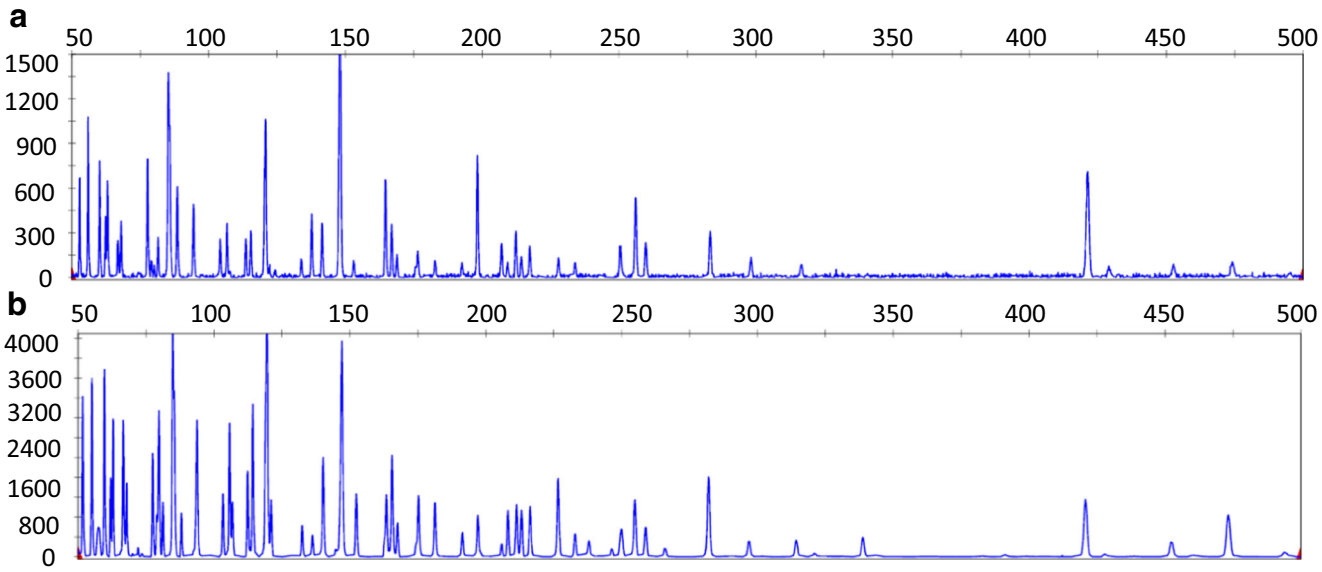


**Fig. 1** Electropherograms of *L. monocytogenes* strain LM44 AFLP profiles. Comparison of AFLP profiles obtained with combined and not-combined digestion-ligation procedure. **a** AFLP profile strain LM44 obtained with not-combined digestion-ligation procedure using AFLP Microbial Fingerprinting kit. **b** AFLP profile strain LM44 performed with combined digestion-ligation procedure in the advanced AFLP protocol

## Pre-amplification

The "non-selective" primers *Eco*RI-0 and *Mse*I-0 (Table 2) were used for pre-amplification of digested-ligated DNA. Each pre-amplification contained 5 μl of digested-ligated DNA previously described, 1.5 μl of unlabelled *Mse*I-0 primer (10 μM), and 1.5 μl of FAM-labeled *Eco*RI-0 primer (10 μM) (BMR Genomics, Padova, Italy) and 25 μl of GoTaq® Colorless Master Mix (PROMEGA, Madison, WI, USA).

The reaction was subjected to the following PCR conditions: 3 min at 94 °C, 14 cycles (45 s at 94 °C, 30 s at 65 °C, and in each cycle the annealing temperature decreased 1 °C, 1 min at 72 °C), 19 cycles (45 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C), 5-min extension at 72 °C, and a final step for 15 min at 30 °C. All amplifications were performed in a GeneAmp® PCR System 2700 (Applied Biosystem, Foster City, CA, USA). Subsequently, the pre-amplification product was diluted 10-fold with RNAse and DNAse free water and stored at − 20 °C.

## Selective amplification

Different primer combinations were used, based on different species analyzed: *Eco*RI-A/*Mse*I-C for *L. monocytogenes* and *Eco*RI-A/*Mse*I-A for *S. thermophilus* and *L. plantarum* (Table 2). Each selective amplification contained 5 μl of the diluted pre-amplification product described previously, 1.5 μl of unlabelled *Mse*I-A/*Mse*I-C primer (10 μM), and 1.5 μl of FAM-labeled *Eco*RI-A primer (10 μM) (BMR Genomics, Padova, Italy) and 25 μl of GoTaq® Colorless Master Mix (PROMEGA, Madison, WI, USA).

The thermocycler program consisted of 2 min at 72 °C, 33 cycles (30 s at 94 °C, 1 min at 56 °C, 1 min at 72 °C), 2-min extension at 72 °C, and a final step for 30 min at 60 °C. All amplifications were performed in a GeneAmp® PCR

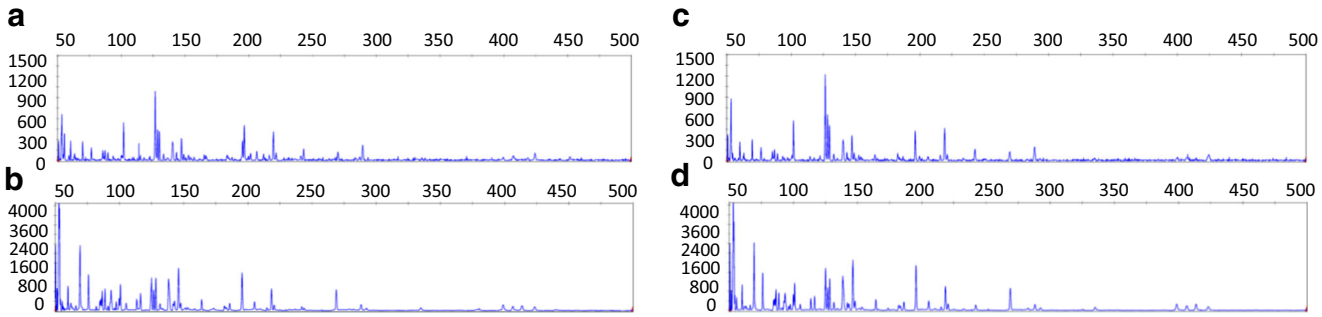


**Fig. 2** Electropherograms of *S. thermophilus* strains 100 and 4042 AFLP profiles obtained using 20-fold and 10-fold dilution of digested-ligated DNA product. **a** AFLP profile of strain 100 related to 20-fold dilution of digested-ligated DNA product. **b** AFLP profile of strain 100 related to 10-fold dilution of digested-ligated DNA product.**c** AFLP profile of strain 4042 related to 20-fold dilution of digested-ligated DNA product. **d** AFLP profile of strain 4042 related to 10-fold dilution of digested-ligated DNA product

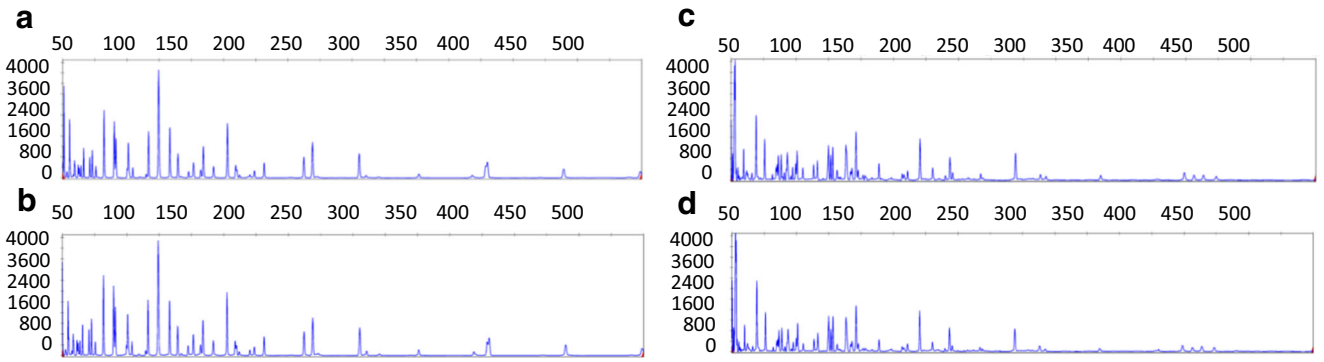


**Fig. 3** Electropherograms of *S. thermophilus* strains 201 and 100 AFLP profiles of the PCR product obtained using 33 and 30 PCR cycles in selective PCR. **a** AFLP profile of strain 201 related to 30 cycles in selective PCR. **b** AFLP profile of strain 201 related to 33 cycles. **c** AFLP profile of strain 100 related to 30 cycles in selective PCR. **d** AFLP profile of strain 100 related to 33 cycles in selective PCR

System 2700 (Applied Biosystem, Foster City, CA, USA). Ten microliters of each selective amplification product were separated by electrophoresis on a 1.2% agarose gel at 90 V/cm for 20 min to confirm the amplifications.

### Fragment analysis

Eight microliters of each amplified products from selective amplification were added to 1.5 μl of GeneScan-500 [LIZ] size standard (Applied Biosystem, Foster City, CA, USA) and 27 μl of deionized formamide. The mixture was heated for 5 min at 95 °C and cooled on ice. Samples were loaded and run on the ABI Prism 310 (Applied Biosystem, Foster City, CA, USA), and analyzed using GeneMapper Analysis Software (Applied Biosystem, Foster City, CA, USA). The data for each run were saved as an individual GeneScan file and displayed as an electropherogram. A threshold, scored to allow only sharp and easily distinguishable peaks, of 50 relative fluorescent unit (RFU) was considered for results obtained by the new protocol, while a threshold of 80 RFU was considered for results obtained with the AFLP Microbial Fingerprinting Kit; all signals under these values were treated as background noise and not scored. Peaks representing AFLP fragments from 50 to 500 bp were reported in a binary format with "1" for presence of a band and "0" for its absence.

## Results

This study compares fragment analysis results obtained with an improved AFLP procedure with the AFLP Microbial Fingerprinting Kit for bacteria AFLP fingerprinting analysis and detection of polymorphisms in bacterial genomes (Zabeau and Vos 1993; Vos et al. 1995; Janssen et al. 1996). The main aim focuses on giving scientific evidence for the possibility to use the improved AFLP protocol to replace the AFLP Microbial Fingerprinting Kit. This protocol also provides an improvement in the profile quality of the assay, increasing sensitivity and precision and decreasing scoring time and errors.

The improved AFLP protocol, in comparison to the AFLP Microbial Fingerprinting Kit, combines the digestion and adaptor ligation at the same time without affecting the number of final bands and the final results. The experiment was performed on the 21 strains shown in Table 1 (Supplementary material), and an example of the result is presented in Fig. 1 where the comparison between the improved protocol and the commercial kit on the strain Lm44 show the same profiles. This procedure, also considered by Curtin et al. (2007), gives the opportunity to save time, by using 4 h of restriction-ligation instead of 24 h of the kit, and to reduce the laboratory costs by six times for the analysis. A second condition that has been also evaluated for modification is the dilution of the digested-ligated DNA

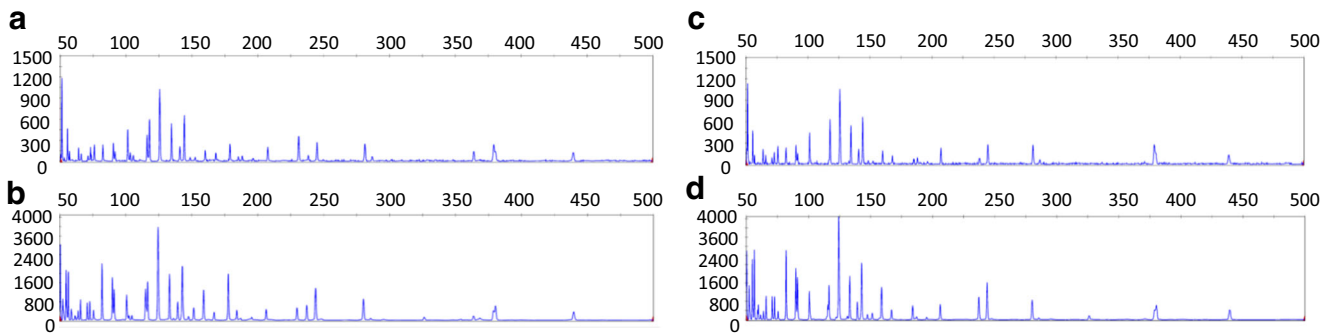


**Fig. 4** Comparisons of the AFLP profiles of *S. thermophilus* strains 4027 and 4028 obtained with the AFLP microbial kit and the improved AFLP protocol. **a** AFLP profile of strain 4027 obtained with the AFLP microbial kit. **b** AFLP profile of strain 4027 obtained with the advanced AFLP protocol. **c** AFLP profile of strain 4028 obtained with the AFLP microbial kit. **d** AFLP profile of strain 4028 obtained with the advanced AFLP protocol

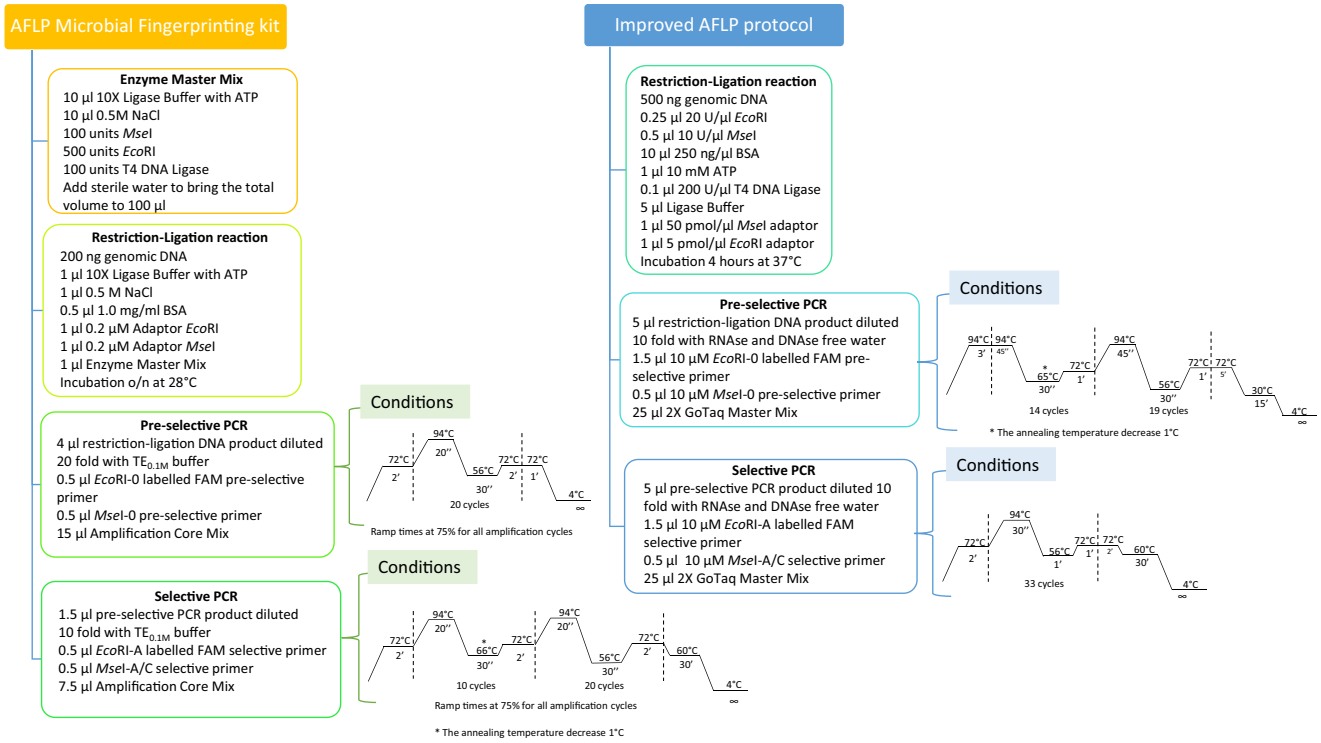


**Fig. 5** Flow chart description of the AFLP Microbial Fingerprinting Kit and the improved AFLP protocol

fragments used as templates in the first amplification reaction. The dilution in the AFLP Microbial Fingerprinting Kit procedure is 20-fold, while in the improved AFLP protocol, this dilution of digested-ligated DNA fragments has a negative influence on the electropherograms resolution, and the optimal dilution of digested-ligated DNA fragments obtained is 10-fold. Figure 2 shows the electropherograms obtained with the two dilutions using the strains *S. thermophilus* 100 and 4042.

The third parameter that was considered for improvement is related to the dilution of the pre-selective PCR products used as templates for the subsequent selective PCR. Two different conditions were evaluated: one without any dilution and one diluted 10-fold. The amplification results analyzed by capillary electrophoresis were similar to one other (data not shown), so the condition reported by AFLP Microbial Fingerprinting Kit and other authors (Vos et al. 1995; Janssen et al. 1996) of the 10-fold dilution was maintained.

Finally, the PCR conditions in the improved protocol have been modified with the touchdown PCR applied only in the pre-amplification reaction and not in the selective one. In addition, the number of PCR cycles in the selective amplification has been taken in consideration with the aim to increase the peak intensity without introducing a high level of *Taq* polymerase errors, which could also give differences in peak base pair size. The number of selective PCR cycles has been increased to 33 compared to Vos et al. (1995), Janssen et al. (1996), and the commercial kit where this parameter was 24 and 30, respectively. The modification introduced allowed us to have electropherograms with more defined and higher fluorescence intensity of peaks for the next step of the data elaboration (Fig. 3). In addition, the optimized protocol provides an improvement in the signal-to-background ratio in the electropherograms and increases the intensity of the peak profiles obtained (Fig. 4 and Supplementary material). Also, the possibility to maintain and compare previous data obtained with the AFLP microbial kit is shown in Fig. 4 where two strains previously analyzed, by Lazzi et al. (2009) using the commercial kit, have the same peak profiles with the improved AFLP protocol; this is also shown in Supplementary material on the other 19 strains. Figure 5 shows a complete description of the two protocols for their comparison and for a prompt use in laboratory.

## Discussion

AFLP is an excellent technique to differentiate strains or very closely related species and is a good phylogenetic tool. In the last years, the combined use of several restriction enzymes and many fluorescence molecules has given opportunities to achieve a very extensive screening of bacteria genomes. The modification of the digested-ligated step drastically reduces the time needed for the sample's analysis. Moreover, the different dilution conditions of digested-ligated DNA fragments and the increase in the number of PCR cycles allow comparable and better results to be obtained in terms of distinctiveness and intensity of the band's peaks when compared to results from the commercial AFLP microbial kit. In addition, considering that the kit is no longer available, the improved AFLP method gives the possibility to compare the AFLP

profiles with previous data without repeating their analyses. This opens the possibility for all laboratories to continue their phylogenetic study using data previously obtained with the AFLP commercial kit. Overall, the modified protocol gives opportunities to reduce the time-consuming and labor-intensive processes, is cost-effective, and maintains the use of the AFLP technique to analyze a large number of samples.

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**Author contributions** Bertani contributed to the analysis, acquisition, and interpretation of data; drafting of the manuscript; and critical revision. Savo Sardaro contributed to the study conception and designs; the work, analysis, and interpretation of data; drafting of the manuscript; and critical revision. Lazzi and Neviani contributed to design the work and in the critical revision.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Animal studies** This article does not contain any studies with animals performed by any of the authors.

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