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Phenotypic and Genotypic Characterization of *Carnobacterium divergens* Isolated from Refrigerated Tunisian Minced Raw Beef Meat

Thouraya Mokrani[1], Ines Essid[1], Mnasser Hassouna[1], Lachheb Jihene[2], Ghram Abdeljalil[2], and Ahlem Jouini[2]*

[1]Unité de Recherche, Food Science and Technology, Higher School of Food Industries of Tunis (ESIAT), University of Carthage, Tunisia
[2]Laboratory of Epidemiology and Veterinary Microbiology, Pasteur Institute of Tunis (IPT), Université de Tunis El Manar, Tunis, Tunisia

Abstract

Six psychotropic strains of *Carnobacterium divergens* were isolated from Tunisian minced raw beef meat packed and stored at 6°C. They were first identified by biochemical methods. Using biochemical reactions and carbohydrate fermentation before their characterisation by molecular techniques. The strain of *Carnobacterium divergens* is a non-motile, Gram-positive psychotropic rod that lacks catalase, oxidase and mannitol. It grows at pH 9.1 (D-MRS agar), but not on acetate agar (pH ≤ 5.4). For all these isolates, the phenotypic identification, using the API 50 CHL system, revealed a variability in the fermentation abilities of some sugars (glycerol, amygdaline, arbutine, D-trehalose and potassium gluconate). Species-specific polymerase chain reaction (PCR) primers were used to ensure identification of these isolated strains at the species level. Moreover, the sequencing of 16S rRNA gene confirmed that all the six isolates are identified as *C. divergens*. The Rep-PCR technique was performed to investigate intra-specific diversity within these six strains of *C. divergens,* commonly identified in meat. The Rep-PCR method distinguished the *C. divergens* strains, demonstrating their biodiversity and their evident similarities. BOX and REP primers allowed amplification with 85% and 80% similarities between the strains, respectively. The BOXA1R and REP primers were proven to be useful for the differentiation of *C. divergens* strains.

Keywords: *Carnobacterium divergens*; Meat; PCR; 16S rRNA; REP-PCR; BOX-PCR

Introduction

Carnobacterium divergens and *C. maltaromaticum* are the only bacteria frequently isolated from a large variety of products like meat [1-5], poultry [6,7] and pork meat juice [8].

Carnobacterium divergens are Gram positive, facultative anaerobic, catalase negative, rod shaped lactic acid bacteria (LAB) which produce L (+)-lactic acid from glucose; they are unable to grow on acetate agar but grow in alkaline environment. They are characterized by the presence of meso-diaminopimelic acid in their cell-wall composition [9,10]. Besides, they are psychotropic bacteria and tolerant to high pressure freezing/thawing. *C. divergens* is often found in products whether they are stored aerobically, vacuum packaged, or subjected to modified conditions storage [1-4,7,11,12]. Raw meat is an extremely perishable proteinaceous food. Its constitute an ecological niche and a potential risk factor for the spread of these pathogens in the environment [13-23]. However, most lactic acid bacteria group, multiply even under vacuum packaged (VP) conditions, do not constitute, from the beginning, in spoiling the meat quality [2,24]. In fact, *C. divergens* have been widely studied in the last two decades as protective cultures, to inhibit pathogenic and spoilage bacteria in food [14-16]. The use of molecular based techniques offers rapid and specific alternatives for species identification. *Carnobacterium* species form a phylogenetically coherent group which different from other LAB, as shown by 16SrDNA sequencing [6,17-20]. Repetitive sequence-based polymerase chain reaction (Rep-PCR) technique was widely employed to characterize bacteria and distinguish clonal diversity [21]. Examples of evolutionarily conserved repetitive sequences are BOX, ERIC, REP and $(GTG)_5$. In Tunisia, the industry of meat conservation is not well developed compared to other economically developed countries. Therefore, there is a particular interest in food product conservations and transformation industries. The objective of this study is the isolation and the identification of *C. divergens* from minced raw beef meat in Tunisia packed and stored at 6°C using a specific gene and the sequencing of 16S rDNA. We identified novel *C. divergens* strains capable to producing bacteriocin variants with a potential application as bio-preservatives. The second purpose was to investigate whether Rep-PCR could be used to analyse such *C. divergens* strains. Besides, two Rep-PCR techniques (BOX and REP-PCR) were compared for their ability to type strains of *C. divergens* and differentiate their genotypes.

Materials and Methods

Sample preparation and lactic acid bacteria isolation

Psychotropic LAB strains were isolated from thirty samples of minced raw beef meat packed and stored at 6°C. The first fifteen meat samples were collected from a market in Tunis and the other ones from a market in Bizerte (North of Tunisia). Strains of psychotropic LAB were isolated on Man Rogosa and Sharpe (MRS) agar (Biokar) adjusted to pH 8.5, with glucose substituted by sucrose and acetate omitted from modified MRS agar [22,23]. All the plates were then incubated at 7°C for 10 days [24] and at least hundred single colonies were randomly picked from D-MRS agar and sub cultivated in Tryptone Soya Broth. The isolates were then sub-cultivated to purity at least twice on D-MRS and incubated at 30°C for 24-48 h.

Phenotypic characterization

Gram staining, cell morphology, oxidase, catalase and mannitol tests were performed using standard procedures according to the methods and the criteria described by Sharpe [25] and Guiraud [24]. The isolates were further characterized by physiological tests which include growth on acetate agar [26], motility, CO_2 production from glucose in D-MRS broth with Durham tubes [27] and oxide fermentation. The ability to ferment carbohydrate substrates was studied using API 50 CHL system

***Corresponding author:** Ahlem Jouini, Laboratory of Epidemiology and Veterinary Microbiology, Pasteur Institute of Tunis, 13 Place Pasteur- BP 74, 1002 Belvédère-Tunis, Tunisia, Tel: 216- 71 783 022; E-mail: ahlem.jouini@pasteur.rns.tn

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(BioMérieux, France). The computer program APIWEB database identification software (BioMérieux) was used to interpret the results. The isolates were stored at -80°C in Tryptone Soya Broth supplemented with 20% glycerol as a cryoprotective agent for further analyses.

Molecular identification

DNA isolation: Bacterial genome DNA was extracted using One-tube Bacterial Genomic DNA Extraction Kit (BIO BASIC CANADA INC). A 0.2 ml volume of fresh overnight culture was transfered into a microcentrifuge tube and centrifuged at 10,000 x *g* for 30 seconds. The pellet was lysed by adding a lysis-buffer containing proteinase K. The sample was incubated at 65°C for 5 min to which a Universal Buffer NST was added. The DNA concentration was measured using a Nanodrop photometric apparatus (Thermo Scientific).

PCR amplification

All used primers were obtained from a commercial supplier (Cartha Genomics Advanced Technologies-Tunisia). A universal forward primer, 27f 5'-AGAGTTTGATCMTGGCTCAG-3', and a reverse primer, Cdi 5'-GCGACCATGCGGTCACTTGAA-3', were used to amplify a small target region (198-199 bp) of the 16S rDNA of *Carnobacterium divergens* [21]. The PCR mixture (50 µl) contained 5 U/µl KAPA Taq DNA Polymerase, 10X KAPA Taq Buffer, 10 mM dNTPs, 10 µM forward and reverse primer, 2 µl genomic DNA and enough sterile deionized water to bring the volume to 50 µl. The mixture was subjected to an initial denaturation step of 3 min at 95°C, followed by 36 cycles of denaturation for 30s at 95°C, annealing for 30s at 55°C, and extension of 1min at 72°C, and a final extension of 1 min at 72°C in a DNA thermal cycler (BIO-RAD T100™). The reaction mixture was visualized on a 2% agarose gel in Tris–acetate buffer containing ethidium bromide (Sigma), and photographed with an UV transilluminator (VILBER LOURMAT, France). The molecular sizes of the amplified DNA fragments were estimated by comparison to a 100-bp DNA ladder (Neo Biotech). The primers used to amplify the whole 16S rRNA gene were fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'- TAAGGAGGTGATCCAGCC-3') [28,29]. The PCR amplified DNA fragments contained the complete 16S rRNA gene; amplification steps consisted of a 60s denaturation at 95°C, a 60s annealing at 56°C and a 90s extension at 72°C. The first cycle was preceded by incubation for 5 min at 94°C. After 35 cycles, a final 7 min extension step at 72°C was realized. The molecular sizes of the amplified DNA fragments were estimated by comparison to a 1 Kb DNA ladder (Thermo Scientific Gene Ruler).

Sequencing and identification of the species

All sequencing reactions were performed in the sequencing unit of "Institute Pasteur of Tunis". The nucleotide sequence of the amplified 16S rRNA gene was determined with an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems using the Big Dye terminator v.3.1 Cycle Sequencing Kit. Anticipated errors of PCR and sequencing reactions were avoided by sequencing both DNA strands. The BLAST algorithm was used to determine the most related sequence relatives in the NCBI nucleotide sequence database (http://www.ncbi.nlm.nih.gov/ BLAST).

Rep-PCR analysis

The repetitive sequence-based oligonucleotide primers (Cartha Genomics Advanced Technologies-Tunisia) used were BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′) and REP (REP1R: 5′-III-ICGICGICATCIGGC-3′, REP2I: 5′-ICGICTTATCIGGCCTAC-3′), each with their specific annealing temperature (1min at 40°C for REP primers and 1min at 52°C for BOX primer). Amplification was carried out in a 25 µl reaction volume, containing 25 µM primer, 5 U/µl KAPA Taq DNA Polymerase, 10X KAPA Taq Buffer, 10 mM dNTPs, 25 mM $MgCl_2$, 5 µl genomic DNA and enough sterile deionized water to bring the volume to 25 µl. The PCR reactions were performed with an initial denaturation step (95°C, 7 min), followed by 35 cycles of denaturation (90°C, 30s), annealing (variable temperature, 1 min) and extension (65°C, 8 min), and a single final extension step (72°C, 16 min) [21]. The amplified fragments were fractionated on a 1% agarose gel during 2-3 h at a constant voltage of 80V in 1X TAE and 0.5 µg/ml ethidium bromide. The Rep-PCR genomic fragments were visualised after staining with ethidium bromide under ultraviolet light, followed by digital capturing of the image using a CCD camera and storage as a tiff file. The resulting fingerprints were analysed using FPQuest ™ software. The similarities among profiles were calculated using the Pearson correlation. Dendograms were constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

Results

Phenotypic identification of isolates

Two hundred and fifty suspected bacteria were isolated on D-MRS agar from the 30 samples of minced raw beef meat packed and stored at 6°C. Those having the following characters: rod shaped cells, Gram positive**,** oxidase negative**,** catalase negative strains. Only sixty isolates were retained. Biochemical identification using the API 50 CH system showed that only six strains were identified as *C. divergens* (Table 1). These isolates showed differences in their fermentation abilities of some sugars. These differences were related to fermentation of glycerol, amygdaline, arbutine, D-trehalose and potassium gluconate (Table 1). The six isolates of *C. divergens* strains were non-motile, facultative anaerobic bacteria, unable to grow on acetate agar and produce CO_2 from glucose.

Molecular identification by and sequencing analyses

For their molecular identification, the six isolates were contained a 199 bp fragment specific to the *C. divergens* strains, with the 27f-Cdi primer pair. The results of the PCR-amplification reactions for presumptive *C. divergens* isolates were consistent with those the biochemical test (Figure 1). Therefore, sequencing of the small target of 16S rRNA gene were indicated that the strains Cd1, Cd2, Cd5 and Cd6 have a high level of sequence homology (reaching 99%) with *Carnobacterium divergens* strain DSM 15683 16S ribosomal RNA gene (NCBI Reference Sequence: NR_113798.1). Strains Cd3 and Cd4 were identified as *C. divergens* showed 99% 16S rRNA sequence homology with *Carnobacterium divergens* strain DSM 20623 16S ribosomal RNA gene (NCBI Reference Sequence: NR_044706.2). To ensure their complete identification, the sequencing of the whole 16S rRNA gene of all isolates was performed to further confirm the identities of the six isolates. In fact, the amplification of 16S rRNA gene showed the presence of one band at almost 1500 bp (Figure 2). Therefore, after sequencing and comparison with the GenBank database, we found that the six identified isolates of *C. divergens* were displayed 99% identity with the 16S rRNA gene of *Carnobacterium divergens* strain DSM 15683 16S ribosomal RNA gene (NCBI ReferenceSequence: NR_113798.1). This is the first report of a molecular approach characterizing *C. divergens* strains from Tunisian minced raw beef meat, taking advantage of the robust sequencing 16S rDNA gene.

Rep-PCR fingerprinting of *Carnobacterium divergens* isolates

DNA fingerprint, produced by Rep-PCR method, provided a clonal diversity of the *C. divergens* strains previously typed at the species level using 16S rDNA sequencing. Rep-PCR primer set (REP1R-I and

Strain	Cd1(87)	Cd2(99)	Cd3(84)	Cd4(98)	Cd5(33)	Cd6(46)
Isolation source	MRM1(E_9)	MRM1(E_{10})	MRM1(E_{10})	MRM1(E_6)	MRM2(E_{18})	MRM2(E_{21})
Gram	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Gaz production	-	-	-	-	-	-
Growth on acetate agar	-	-	-	-	-	-
Sugars fermented (API 50 CH):						
Glycerol	+	+	+	-	-	+
D-Ribose	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+
N-Acetylglucosamine	+	+	+	+	+	+
Amygdaline	+	-	-	-	+	+
Arbutine	+	+	-	+	+	+
Salicine	+	+	+	+	+	+
D-Celiobiose	+	+	+	+	+	+
D-Maltose	+	+	+	+	+	+
D-Saccharose(sucrose)	+	+	+	+	+	+
D-Trehalose	+	-	-	+	+	+
D-Melezitose	+	+	+	+	+	-
Gentiobiose	+	+	+	+	+	+
Potassium Gluconate	+	-	-	-	-	+

Cd: Carnobacterium divergens.
MRM: sample of ‘‘Minced Raw Beef Meat”.
MRM 1: samples were taken from market in Tunis; MRM 2: samples were taken from in Bizerte.
E: sample number from 1 to 30.
+: positive; -: negative.
None of the strains of Carnobacterium divergens was able to ferment: Erythritol, D-arabinose, L-Arabinose, D-xylose,L-xylose D-adonitol, Methyl-BD-xylopyranoside, D-galactose, D-sorbose, L-rhamnose, Dulcitol, Inositol, D-manitol, D-sorbitol, Methyl-αD-Mannopyranoside, Methyl-αD-Glucopyranoside,Esculine citrate de fer, D-lactose, D-melibiose, Inulin, D-raffinose,Amidon, Glycogen, Xylitol, D-Turanose, D-lyxose,D-Tagatose, D-fucose, L-fucose, D-Aarabitol, l-Aarabitol, Potassium2-ketogluconate, Potassium5-ketogluconate

Table 1: Origin and biochemical profiles of the six *Carnobacterium divergens* strains isolated from Tunisian minced raw beef meat packed at 6°C.

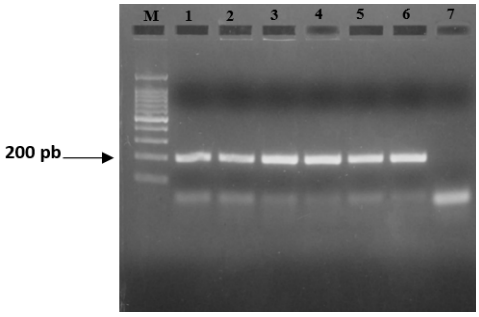

Figure 1: PCR products of *Carnobacterium divergens* strains, obtained using small 16S rRNA-targeted, species-specific primers (27f-Cdi primer pair); Lane labelled M; 100-bp DNA ladder, lanes (1, 2, 3, 4, 5 and 6); Cd1, Cd2, Cd3, Cd4, Cd5 and Cd6, respectively (Cd: *Carnobacterium divergens*), lane 7; negative control. Lane numbers are indicated in bold.

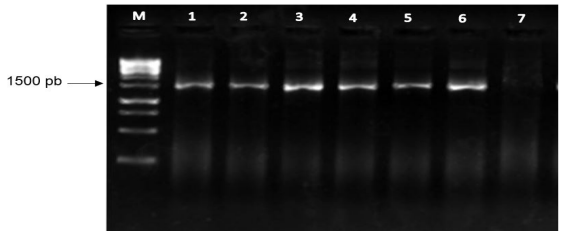

Figure 2: PCR products from *Carnobacterium divergens* strains, obtained using 16S rRNA-targeted, specific primers (Fd1-Rd1 primer pair); Lane labelled M; 1 Kb DNA ladder, lanes (1, 2, 3, 4, 5 and 6); Cd1, Cd2, Cd3, Cd4, Cd5 and Cd6 respectively (Cd: *Carnobacterium divergens*), lane 7; negative control. Lane numbers are indicated in bold.

REP2-I primers) and BOXA1R primer were used to compare the clonal diversity of the six closely related strains of *C. divergens*. Each primer generated profiles that vary within the same species and differ in the number and the size of produced bands. Although Rep-PCR revealed similar patterns for the six studied strains, the REP-PCR and BOX-PCR methods showed a better discriminatory power. For *C. divergens*, the isolates were grouped in clusters, demonstrating their biodiversity and, at the same time, their similarities with isolates within the same species. The REP set and the BOX primer, gave successful amplification with a total of 10-12 and 8-12 bands having sizes lying between 250 bp-3500 bp and 350 bp-4500 bp, respectively. In comparison with the BOXA1R primer, the REP1R-I and REP2-I primer set clearly generated more bands which allowed easy interpretation and visual differentiation. Each strain gave almost a different REP-PCR fingerprinting although common two intense bands, between 2000 bp and 1500 bp, were observed for all six strains, and along with four bands of 400, 800, 900 and 3500 bp.

A dendrogram was constructed showing the similarities among the different profiles using the cluster analysis software (Figure 3). At 80% coefficient value, three distinct clusters could be delineated. According to dendrogram analysis, cluster 1 was grouped two strains of *C. divergens* (Cd3 and Cd6) with 85% similarity. Two *C. divergens* (Cd2 and Cd4), sharing 98% similarity, were clustered with the (Cd1) strain which shares 94% similarity and all three-formed cluster 2. The strain (Cd5) formed a distinct cluster with a very low similarity level of 40%. The amplification profiles from the six identified *C. divergens* gave only a very intense common band, slightly above 1000 bp. Three other common bands were also observed, the first one at 350 bp, the second between 1000 and 1500 bp and the third one slightly below 2000 bp. The

constructed dendrogram showed the similarities among the different profiles using the cluster analysis software (Figure 4). The six strains were classified according to their BOX-PCR profiles into 4 clusters, at a similarity level of 85%. According to unweighted average method, using amplification profiles obtained for *C. divergens* strains; cluster 1 grouped 2 strains of *C. divergens* (Cd3 and Cd6) at 87% similarity, cluster 2 contained only one *C. divergens* strain (Cd2) at a similarity level of 77%, cluster 3 grouped two strains of *C. divergens* (Cd1 and Cd4) sharing 87% similarity and cluster 4 contained one strain (Cd5) at a similarity level of 81%.

Discussion

The diversity of LAB in Tunisian meat products, especially in minced raw beef meat, depends on adventitious microflora present in the meat and meat environment as well as on a number of selective conditions persisting during the manufacturing process. In this study, we report the isolation and identification of *C. divergens* strains. They were isolated from Tunisian minced raw meat packed, stored at 6°C then commercialized for human consumption. The catalase negative, oxidase negative and mannitol-negative *C. divergens* isolates, are psychotropic, microaerophilic but oxygen-tolerating bacteria, able to grow in a wide variety of refrigerated raw and processed meat products stored aerobically. They survive freezing and are able to grow in meat after thawing. They are weakly acid tolerant which can be associated with spoilage of refrigerated meat and fish products [6,30,31]. Fresh meat is easily contaminated by the slaughtering process and serves as a substrate for different spoilage and pathogenic bacteria, it harbours non-negligible health risks for all end consumers. However, it could be shown that *C. divergens* strain is frequently present in fresh meat products, but absent in spoiled products [2,32]. *Carnobacteria* differ from most lactobacilli as they are able to proliferate at low temperatures. In addition, *Carnobacteria* may be distinguished from lactobacilli by their inability to grow in acetate containing media at pH values ≤ 5.6, but are able to grow well in media, at an alkaline pH around 9.1. The phenotypic tests provided some evidence of metabolic capabilities but there were some problems related to a lack of reproducibility and discriminatory power. None of the phenotypic tests included in the present study clearly differentiated between *C. divergens,* at the species level. Clearly, *C. divergens* strains were identified by PCR assays using 16S rDNA-directed primers. Therefore, we have used 16S rRNA gene sequencing of all isolated *C. divergens* strains to enhance their detection in minced raw meat. Thus, universal primers (27f-Cdi) were used to amplify target regions (198-199 bp) of the 16S rDNA gene which is considered as a reliable and a useful target for *C. divergens* strain identification. In the present study, we also used the sequencing of the whole 16S rRNA gene as described by Kabadjova [33] and confirmed its ability to identify our six isolates of *C. divergens* at the species level. High degrees of similarity (reaching 99%) of the 16S rRNA gene sequences were observed among the six closely related *C. divergens.* Indeed, these strains demonstrated high identity (99%) with the 16S rRNA gene of *C. divergens* strain DSM 15683. Based on the phenotypic characters and the API system, our results confirmed the identification of the six selected strains. These strains shared very similar 16S rRNA gene sequences (99%) and demonstrated similar phenotypic traits making it difficult to differentiate them. For this reason, the reproducibility and the discriminatory power of Rep-PCR method in typing strains at the intra-species level, proved to be useful for genotypic fingerprinting and grouping of the *C. divergens* strains. In fact, Rep-PCR using the primers BOXAIR and REP has allowed strain differentiation at the intra-species level [34,35]. To date, no studies are available on the use of the Rep-PCR fingerprinting to discern the intra-species diversity of *C. divergens* isolated from food, especially meat or meat products. In our case, the more important discriminatory power was obtained when using the BOXA1R primer, which generates banding patterns displaying higher intra-species heterogeneity as compared to REP-generated banding patterns. These results confirmed those reported by Masco [34], regarding *Bifidobacterium* species, even though the REP-PCR profiles were easy to interpret, can sufficiently differentiate the isolates and showed strain specific bands. To our knowledge, no studies have been reported on the use of the BOXA1R primer and the REP primer set for Rep-PCR fingerprinting of *C. divergens* strains. In this regard, we have demonstrated the usefulness of Rep-PCR fingerprinting with the REP and BOX primers for typing *C. divergens* strains. Besides, this study described, for the first time, the presence of *C. divergens* species during the cold storage of Tunisian minced raw beef meat packed and stored at 6°C, at an industrial scale. The minced raw beef meat contained *C. divergens* species that exist in such environment. Our results of this study brought new information about the diversity of *C. divergens* species in our region. They also suggested that Tunisian minced raw beef meat could be a source of *C. divergens* with considerable differences in respect to acidifying and other technological activities which are being demonstrated. The use of such strains in meat industry could offer great potential for new industrial applications or existing processes. Selected *C. divergens* with slow acidification rate and important technological activities, especially production of bacteriocin, could be used as adjunct cultures for improving the bio preservative, under controlled conditions, of meat product processing.

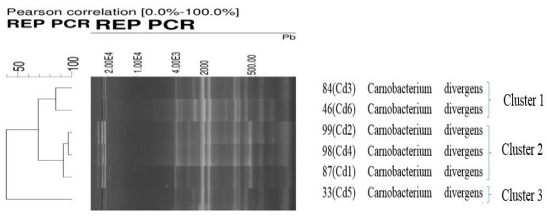

Figure 3: Dendrogram generated from REP-PCR fingerprints of the six *C. divergens* strains obtained from minced raw beef meat. The dendrogram was constructed using the unweighted pair group method using arithmetic averages with correlation levels expressed as percentage.

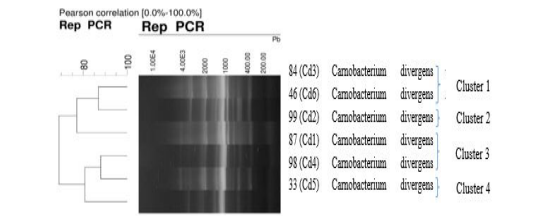

Figure 4: Dendrogram generated from BOX-PCR fingerprints of the six *C. divergens* strains obtained from minced raw beef meat. The dendrogram was constructed using the unweighted pair group method using arithmetic averages with correlation levels expressed as percentage.

Conclusion

The study described, for the first time, the presence of *C. divergens* species during cold storage at 6°C of Tunisian minced raw beef meat, which is manufactured on the industrial scale; therefore, they might contain such species found in their environment. The results of phenotypic methods for bacterial identification might be difficult to interpret or ambiguous and required subsequent confirmation. Combination of phenotypic and genotypic methods is considered beneficial for the most reliable identification. Species-specific PCR-based protocols were carried out for the identification of each *C. divergens* species. At the end, the results of our study offered new

information about the presence of *C. divergens* species in our region. The study recommended that REP-PCR and BOX-PCR are a highly discriminatory technique that permit differentiation at the intra-species and potentially up to the strain levels. Moreover, isolated and characterized *C. divergens* strains could be eventually used for the construction of starter cultures for bio preservation of minced raw beef meat under controlled conditions in a meat factory.

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