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Genotypic and phenotypic characterization of the food spoilage bacterium *Brochothrix thermosphacta*

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ABSTRACT

Microbial food spoilage is responsible for significant economic losses. *Brochothrix thermosphacta* is one of the major bacteria involved in the spoilage of meat and seafood. Its growth and metabolic activities during food storage result in the production of metabolites associated with off-odors. In this study, we evaluated the genotypic and phenotypic diversity of this species. A collection of 161 *B. thermosphacta* strains isolated from different foods, spoiled or not, and from a slaughterhouse environment was constituted from various laboratory collections and completed with new isolates. A PCR test based on the *rpoB* gene was developed for a fast screening of *B. thermosphacta* isolates. Strains were typed by MALDI-TOF MS, rep-PCR, and PFGE. Each typing method separated strains into distinct groups, revealing significant intra-species diversity. These classifications did not correlate with the ecological origin of strains. The ability to produce acetoin and diacetyl, two molecules associated with *B. thermosphacta* spoilage, was evaluated in meat and shrimp juices. The production level was variable between strains and the spoilage ability on meat or shrimp juice did not correlate with the substrate origin of strains. Although the *B. thermosphacta* species encompasses ubiquitous strains, spoiling ability is both strain- and environment-dependent.

1. Introduction

Brochothrix thermosphacta is recognized as the dominant food spoiler of meat and seafood products stored under modified atmosphere packaging (Remenant et al., 2015). This ubiquitous microorganism has been isolated from foods of animal origin such as meat, seafood and dairy products (Stackebrandt and Jones, 2006). Moreover, it has been described as widely disseminated along the food chain, from the raw material to the final product, as well as in the food processing environment (Nychas et al., 2008; Stackebrandt and Jones, 2006). *B. thermosphacta* can cause serious economic losses in the food industry due to its ability to produce metabolites associated with off-odors. For example in beef meat, it has been shown to produce cheesy and creamy dairy off-odors associated with the production of 3-hydroxy-2-butanone (acetoin), 2,3-butanedione (diacetyl), and 3-methyl-1-butanol (Casaburi et al., 2014; Dainty and Mackey, 1992). In cold-smoked salmon, *B. thermosphacta* produces 2-hexanone and 2-heptanone, two compounds responsible for the formation of the blue-cheese off-odor (Joffraud et al., 2001; Laursen et al., 2006; Mejlholm et al., 2005). Strong butter, buttermilk-like, sour, and nauseous off-odors caused by *B. thermosphacta* in cooked and peeled shrimp have been associated with the production of 2,3-butanedione (diacetyl), 3-methyl-1-butanal, and 3-methyl-1-butanol (Jaffrès et al., 2011; Laursen et al., 2006; Mejlholm et al., 2005). Therefore, the molecules produced by *B. thermosphacta* seem to depend on the food matrix. However, the above-mentioned studies used different strains and thus it is possible that the spoilage potential is also strain-dependent.

B. thermosphacta is a facultative anaerobe that can grow on chilled meats and fish stored under low O_2 and under vacuum

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packaging (Borch et al., 1996; Drosinos and Nychas, 1997; Ercolini et al., 2006). Although glucose is not present at high concentration in meat, Gill and Newton (1977) reported that it is the preferred substrate of *B. thermosphacta* when grown in meat juice. In addition glucose metabolism is greatly affected by the composition of the gas used for storage. Under aerobic conditions, 3-hydroxy-2-butanone (acetoin) and 2,3-butanedione (diacetyl) are the major metabolites produced by the consumption of glucose while under anaerobic conditions, *B. thermosphacta* produces lactic acid and ethanol (Dainty et al., 1985; Pin et al., 2002). Thus, spoilage activity may also vary depending on food storage conditions.

Brochothrix and *Listeria* genera constitute the *Listeriaceae* family, and the *Brochothrix* genus encompasses two non-pathogenic species: *B. thermosphacta* and *Brochothrix campestris*. However, little information is available for *B. campestris*, most of the available information refers mainly to a single strain (ATCC 43754, the type strain) (Gribble and Brightwell, 2013; Talon et al., 1988).

Various molecular techniques have been widely applied to genotype foodborne pathogenic or spoilage bacterial species. Pulsed Field Gel Electrophoresis (PFGE) has been described as highly discriminatory, robust and reproducible (Gerner-Smidt et al., 2006; Graves and Swaminathan, 2001; Lukinmaa et al., 2004). It has been used successfully for genotyping *B. thermosphacta* meat isolates (Papadopoulou et al., 2012). Repetitive-element Palindromic PCR (rep-PCR) has been widely applied for molecular typing and has proven to be a powerful tool in environmental and food microbiology (Ishii and Sadowsky, 2009). It can differentiate a wide range of bacterial species at the subspecies or even the strain level (Wolska and Szweda, 2012) and has been applied to the differentiation of *B. thermosphacta* (Papadopoulou et al., 2012; Xu et al., 2010). Recently, various studies have also shown the applicability of Matrix Assisted Laser Desorption/Ionization Time Of Flight Mass Spectrometry (MALDI-TOF MS) for bacterial identification, taxonomy and strain typing (Singhal et al., 2015). Widely used in clinical microbiology for identification purposes (Carbonnelle et al., 2011), this technique is an effective tool for the intra-specific typing of bacteria from the genera *Listeria* (Barbuddhe et al., 2008) and *Salmonella* (Dieckmann et al., 2008) and is increasingly applied for identifying and typing microorganisms associated with food (Böhme et al., 2011; Kern et al., 2014).

Several studies have led to different conclusions about the intra-species diversity of *B. thermosphacta* (Papadopoulou et al., 2012; Stanborough et al., 2017). This may be due to the small number of strains, the low diversity of the ecological origin of the studied collections, or the use of different methods. In addition, the genetic functions involved in spoilage remain little studied. The analysis of draft genome sequences of 13 *B. thermosphacta* strains pointed out some genes potentially involved in spoilage activity, but did not reveal any strong diversity (Stanborough et al., 2017). Therefore, genetic diversity and its potential link with the ecological niches or spoilage ability of *B. thermosphacta* remain unsolved.

In order to investigate whether a correlation can be established between the ecological origins of *B. thermosphacta* strains, their diversity and their spoilage potential, we constituted a collection of strains from a wide range of ecological environments. MALDI-TOF MS, PFGE, and rep-PCR were used to assess diversity in the collection while phenotypic diversity was evaluated through the quantification of acetoin and diacetyl production, two molecules associated with spoilage by *B. thermosphacta*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The 161 *B. thermosphacta* strains isolated during this study (N = 80) or sourced from various collections (N = 81) are listed in Table 1. In addition, *B. campestris* ATCC 43754 (= DSM 4712), *Listeria innocua* ATCC 33090, *L. innocua* CLIP 11262, *L. monocytogenes* ATCC 35152, *L. monocytogenes* 08−5578 (Gilmour et al., 2010), *Carnobacterium maltaromaticum* ATCC 27865, *Carnobacterium divergens* V41 (Pilet et al., 1995), *Staphylococcus epidermidis* ATCC 12228, *S. epidermidis* RP62A (Gill et al., 2005), *Serratia liquefaciens* ATCC 27592, and *Escherichia coli* K12 were used as controls for various purposes. Bacteria were routinely grown in BHI broth (VWR Chemicals, France) at 25 °C for *Brochothrix* sp., 30 °C for *Listeria* sp., *Carnobacterium* sp., *S. epidermidis*, and *S. liquefaciens*, and at 37 °C for *E. coli*.

Minced beef meat and peeled shrimp juices were used as broth to quantify acetoin and diacetyl production by *B. thermosphacta* strains. Meat juice was prepared by stomaching ground beef, collected frozen from a local supermarket, as previously described by Rantsiou et al. (2012), and filtered through a 0.45 μm membrane filter before sterilization with a 0.2 μm membrane filter. Shrimp juice was prepared by crushing frozen raw peeled shrimp from Ecuador (91/100 without sulfite, purchased from industry, Nantes) in sterile distilled water. The shrimp based mixture was heated (100 °C; 2 min), filtered and autoclaved (100 °C; 30 min) as previously described by Fall et al. (2010). Five milliliter aliquots were then stored frozen at −20 °C in 15 ml tubes until use.

Bacterial enumeration was performed after 48 h of incubation on Plate Count Agar (PCA) (Biomerieux, France) at 30 °C and *B. thermosphacta* selective STAA agar base containing STAA selective supplement (Oxoid, France) at 25 °C, to determine the total aerobic and *B. thermosphacta* counts, respectively.

2.2. Sampling new B. thermosphacta isolates

Sampling was carried out in a beef slaughterhouse at five points: (i) the chilling room (walls and floors), (ii) the nacelle receiving the viscera, (iii) the knives used for skinning, (iv) animal skin and (v) cattle barns (floor and walls). About 10 cm^2 of knife surfaces were sampled by rubbing cotton swabs five times in both vertical and horizontal directions for 30 s. Other surfaces (walls, floors, nacelles, skin) were sampled using sterile wipes impregnated with peptone water. Samples were transported from the collection site to the laboratory in a cooler (4 °C) and analyzed immediately. Swab and wipe samples were homogenized by shaking manually with 10 ml and 25 ml of peptone water (Biokar Diagnostics, France), respectively. New isolates from ground beef meat and chicken cuts were also collected in the present study. Chicken cuts were rinsed in peptone water as previously described (Rouger et al., 2017) whereas beef meat was stomached in 0.9% NaCl solution for 3 min. Then, appropriate decimal dilutions were plated on PCA and STAA plates for bacterial enumeration. Three to four colonies were selected from STAA plates, then purified on Brain Heart Infusion (BHI) agar (VWR Chemicals, France) and stored at −80 °C in BHI broth supplemented with 20% (v/v) glycerol (VWR Chemicals, France).

2.3. DNA extraction

DNA was extracted from 2 ml of overnight cultures with the DNeasy blood and tissue kit (Qiagen, France) according to the manufacturer's instructions. DNA concentration and purity were estimated after electrophoresis on 1% agarose. DNA extracts were stored at −20 °C.

2.4. rpoB species-specific primer design and PCR conditions

A PCR primer set was designed to amplify a DNA fragment

Table 1
B. thermosphacta isolates used in this study.

Ecological origin (a)	Strains	Laboratory collection	Reference
Beef slaughterhouse environment (7)	BSAS1 1, BSAS1 3, BSAS2 4, BSBS1 3, BSBS1 6, BSAS2 3, BSK1 3	INRA-SECALIM	This study
Chicken legs (48)	TAP 54, TAP 56, TAP 57, TAP 58, TAP 61, TAP 62, TAP 63, TAP 64, TAP 68, TAP 69, TAP 73, TAP 74, TAP 76, TAP 78, TAP 81, TAP 104, TAP 108, TAP 109, TAP 110, TAP 111, TAP 123, TAP 125, TAP 129, TAP 105, TAP 107, TAP 126, TAP 142, TAP 143, TAP 144, TAP 146, TAP 147, TAP 148, TAP 164, TAP 166, TAP 168, TAP 169, TAP 170, TAP 171, TAP 172, TAP 175, TAP 176, TAP 180, TAP 199, TAP 201, TAP 202, TAP 203, TAP 204, TAP 206, TAP 207	INRA-SECALIM	This study
Beef meat (25)	VHB2, VHB3, VHU1, VHU2, VHU3, VHF DLC1 1, VHF DLC1 2, VHF DLC1 3, VHF DLC2 1, VHF DLC2 2, VHF DLC2 3	INRA-SECALIM	This study
	V2, G8, G6, G7, MFPA17A17-02, MFPA19A15-05, MFPA22A14-04, MFPA22A14-05, MFPA42A14-07, MFPA43A14-06, MFPB17A13-02, MFPB42A12-05, MFPB43D06-02, MFPB43A12-01	INRA-MICALIS/FME	(Lucquin et al., 2012)
Lamb meat (1)	8727		
Horse meat	160X7, 160X8		
Beef and lamb sausages (4)	M1, M2, M4, M6		
Pork meat (10)	DSM 20171T = ATCC 11509, DSM 20599	DSM/ATCC	(McLean and Sulzbacher, 1953; Sneath and Jones, 1976)
	FMCC B-427, FMCC B-428, FMCC B-429, FMCC B-430, FMCC B-431, FMCC B-432, FMCC B-433, FMCC B-434	LFMB/Agricultural University of Athens	
Shrimps (20)	CD 251, CD 252, CD 266, CD 274, CD 280, CD 290, CD 321, CD 322, CD 326, CD 331, CD 337, CD 340, CD 350, CD 352, CD 355, CD 357, CD 358, CD 372, CRE 2330, CRE 2333	INRA-SECALIM/ IFREMER-EM3B	(Jaffrès et al., 2009)
Cod fillet (4)	EBP 3017, EBP 3018, EBP 3032, EBP 3033	IFREMER-EM3B	(Chaillou et al., 2015)
Salmon (30)	EBP 3069, EBP 3070, EBP 3083, EBP 3084, SF 677, SF 678, SF 711, SF 712, SF 713, SF 746, SF 748, SF 750, SF 779, SF 781, SF 782, SF 1173, SF 1186, SF 1216, SF 1234, SF 1820, SF 1838, SF 1849, SF 1926, SF 1930, SF 1939, MIP 2440, MIP 2490, MIP 2576, MIP 2599, MIP 2622	INRA-SECALIM/ IFREMER-EM3B	
Sea bream (8)	FMCC B-112, FMCC B-113, FMCC B-114, FMCC B-115, FMCC B-116, FMCC B-117, FMCC B-118, FMCC B-119	LFMB/Agricultural University of Athens	
Cheese rind (1)	cH8.14	INRA-URF	(Almeida et al., 2014)
Unknown (1)	5X10003	INRA-MICALIS/FME	

a: Number of strains.
IFREMER-EM^{3}B: French Research Institute for Exploitation of the Sea, Nantes, France.
INRA-MICALIS/FME: UMR INRA/AgroParisTech (Microbiologie de l'alimentation au service de la santé), Jouy en Josas, Paris, France.
INRA-SECALIM: UMR INRA/Oniris (Sécurité des Aliments et Microbiologie), Nantes, France.
INRA-URF: Unité de Recherches Fromagères, INRA Aurillac, France.
LFMB: Laboratory of Food Microbiology and Biotechnology, Agricultural University of Athens, Greece.

specific to *B. thermosphacta,* excluding *B. campestris*, other closely related species (such as *Listeria*) and species present in the same environments or reported as growing on STAA medium (such as *Carnobacterium,* or *Staphylococcus* sp.). The *in silico* primer design was based on the multiple alignment of *rpoB* gene sequences available from the GenBank database. The *rpoB* sequences of *B. thermosphacta* ATCC 11509, *B. campestris* ATCC 43754, and the most closely related bacterial species were aligned using the BioEdit-ClutalW Sequence Alignment program (Hall, 1999). Specific primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/). The *in silico* specificity of primers was assessed by nucleotide BLAST [National Center for Biotechnology Information (NCBI)] analysis and Primer3. Oligonucleotides with the highest specificity for the *B. thermosphacta* sequence, without hairpin structures or dimers, were selected. This resulted in the design of the forward primer (*rpoB*-Fw1_154–175: 5′-GCGTGCATTAGGTTTCAGTACA-3′) and the reverse primer (*rpoB*-Rev1_525–547: 5′- TCCAAGACCAGACTCTAATTGCT-3′) for the specific amplification of 394 bp of the *B. thermosphacta rpoB* gene. Primer specificity was then assessed by PCR amplification on the DNA extracted from *B. campestris*, *Listeria* sp, *Carnobacterium* sp, and *Staphylococcus* sp.

Amplifications were performed in a 50 µl reaction volume containing: 1 µl (50–100 ng) of DNA, 5 µl of 10X *Taq* Buffer (New England Biolabs, France), 0.2 µM of dNTP (New England Biolabs, France), 0.4 µM of each primer and 1.5 U of *Taq*-polymerase (New England Biolabs, France). PCR reactions were carried out with a PTC-100 Thermocycler (Bio-Rad Laboratories, France) using the following amplification conditions: initial denaturation step at 95 °C for 5 min, followed by 25 cycles of [denaturation (95 °C for 30 s), primer annealing (66 °C for 30 s), primer extension (72 °C for 30 s)], and a final extension step at 72 °C for 5 min. Amplicons were separated in a 1.5% (w/v) agarose gel containing 0.05X of Syber Safe (Invitrogen Life Technologies, France) in TAE buffer. The gel was visualized under UV transillumination (Bio-Rad Laboratories, France).

2.5. 16S rDNA sequencing

The 16S rDNA (about 1500 bp) was amplified by PCR according to Jaffrès et al. (2009). Fragments were partially sequenced (about 800 bp) using the Eurofins Genomics service (Les Ulis, France). The resulting sequences were cleaned then assembled into a unique contig sequence with BioEdit software (Hall, 1999). A BLAST search of partial 16S rRNA gene sequences was performed in the NCBI database (NCBI, Bethesda, USA).

2.6. MALDI-TOF

For MALDI-TOF MS analysis, fresh cultures of *Brochothrix* sp. incubated at 25 °C were centrifuged (10 min; 3000 *g*; 4 °C) and rinsed in 1 ml of molecular biology grade water. One microliter of the bacterial suspension was spotted in a square-form onto the sample target plate in 8 replicates (Bruker Daltonics, Germany) and allowed to dry in a biosafety cabinet at ambient temperature. Each droplet was overlaid with 1 µl of HCCA matrix solution, a saturated solution of alpha-cyano-4-hydroxy cinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid (Bruker Daltonics, Germany), and

then dried as above. As a positive control for each run and for calibration purposes, one spot was also covered by the Bacterial Test Standard (BTS) mixture (Bruker Daltonics, Germany). Measurements were made using the manufacturer's recommended settings (linear positive mode, Nitrogen Laser with 60 Hz repetition rate, 20-kV acceleration voltage, 18.5-kV IS2 voltage, 250 ns extraction delay, and 2000 to 20,000 m/z range). For each sample, mass spectra were examined visually using FlexAnalysis (Bruker Daltonics V3.4) to identify large spot-to-spot inconsistent variations. Spectra were then imported into a Matlab (MathWorks) script, which performs smoothing, normalization, baseline subtraction and peak selection automatically. From a selected peak list associated to intra-species variations, a dendrogram was generated using the Euclidean distance measure and an average linkage.

2.7. Rep-PCR

DNA was subjected to rep-PCR analysis according to Ouoba et al. (2008) using (GTG)5 primer (5′-GTGGTGGTGGTGGTG-3′). Amplicons were separated in a 2% (w/v) agarose gel in 1 x TAE at 3 V/cm for 3 h. After the run, gels were stained with 0.5 μl/ml Syber Safe (Invitrogen Life Technologies, France) for 1 h and then visualized with UV transillumination (Bio-Rad Laboratories, France). DNA profiles were analyzed with Bionumerics software, version 6.5 (Applied-Maths, Belgium). Isolates were compared using the band-based Dice coefficient (optimization: 0.5%; tolerance: 1%) and UPGMA (unweighted pair-group method using the average approach) cluster analysis.

2.8. PFGE

Genomic DNA from 2 ml of overnight cultures was prepared in low-melting-point agarose plugs as described by Doulgeraki et al. (2010), and digested with the endonuclease *Apa*I (New England Biolabs, France) according to the manufacturer's instructions. Electrophoresis was performed on the CHEF-DRIII PFGE system (Bio-Rad Laboratories, France) in 1% (w/v) agarose gels with 0.5 x TBE as the running buffer, at 14 °C. A lambda ladder (Bio-Rad Laboratories, France) was used as the molecular weight marker. Restriction fragments were resolved at a constant voltage of 6 V/cm with switch times of 4–40 s for 18 h and 4–12 s for 4 h. Gels were stained with 0.5 mg/ml ethidium bromide, and DNA bands were visualized with UV transillumination (Bio-Rad Laboratories, France). PFGE profiles were analyzed using the BioNumerics Software, version 6.5 (Applied-Maths, Belgium), and then compared using the Pearson coefficient (optimization: 0.5%; curve smoothing: 0%) and UPGMA cluster analysis.

2.9. Acetoin/diacetyl production

2.9.1. Voges-Proskauer reaction

Tests were performed in 48-well plates (Falcon, France) on 0.5 ml samples pipetted from 10 ml cultures collected after 48 h of growth at 25 °C and gentle resuspension of cells. A volume of 75 μl of alpha-naphthol (5% (w/v) in 95% ethanol) and 50 μl of KOH [40% (w/v) in water] was added. Plates were incubated at room temperature for 1 h. The level of acetoin production was assessed visually using a six-point scale based on the color intensity and noted from (0): not produced or light yellow to (5): very high production or strong red color.

2.9.2. Acetoin and diacetyl quantification

Five milliliter aliquots of meat or shrimp juice were gently defrosted at 4 °C and then inoculated (1:100) with overnight cultures grown at 25 °C in BHI broth. After incubation for 48 h at 25 °C, 2 ml aliquots were centrifuged for 10 min at 10,000 *g* and the supernatant was recovered for acetoin and diacetyl quantification as described by (Nicholson, 2008; Westerfeld, 1945). The reaction was carried out on 200 μl of culture supernatant by the addition of 140 μl of creatine [0.5% (w/v) in water], 200 μl of alpha-naphthol [5% (w/v) in 95% ethanol], and 200 μl of KOH [40% (w/v) in water]. A blank was prepared simultaneously with non-inoculated meat and shrimp juices. Absorbance at 560 nm of the samples was measured in a spectrophotometer (Spectronic Genesys 5) after incubation at room temperature for 10 min (for diacetyl) and 1 h (for acetoin). Standard curves were constructed with controls containing increasing acetoin and diacetyl concentrations and used to calculate acetoin and diacetyl production. pH was measured at the end of the experiments using a Crison pH-meter (Crison micro pH 2000, Spain). Analyses were performed in triplicate.

2.10. Statistical analysis

2.10.1. Hierarchical clustering and multidimensional scaling

Similarities between isolates were first converted into dissimilarities by complement to 1 of the Dice coefficient (for rep-PCR) and the Pearson coefficient (for PFGE). These dissimilarities were analyzed using two different statistical techniques: the hierarchical clustering of isolates and the factorial representation of individuals by Multidimensional Scaling (MDS).

Dendrograms of isolates were obtained by hierarchical clustering of the two matrices of dissimilarities. The agglomerative procedure was the UPGMA, also known as the group average linkage (Everitt et al., 2001).

Multidimensional scaling aims to derive a factorial representation of individuals from a measure of the dissimilarity between them (Borg and Groenen, 2005). The two matrices of dissimilarities between isolates were summed and submitted to MDS in order to produce a spatial configuration representing the distances between isolates. The quality of fit was measured by the stress index, which is the sum of the squared differences between the initial dissimilarities and distances in the configuration. The obtained stress value was 0.147 with 6 dimensions, indicating an acceptable goodness of fit of the MDS configuration (Krzanowski, 1990). The final configuration was rotated in order to interpret the results more easily.

Data were statistically analyzed with the R packages *cluster* (for hierarchical clustering) and *smacof* (for multidimensional scaling).

2.10.2. Analysis of variance

Analysis of variance (ANOVA) with R version 3.3.2 (C) 2016 (The R Foundation for Statistical Computing) was performed to determine statistically significant differences between strains grown in meat and shrimp juices. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Constitution of a B. thermosphacta strain collection

As the purpose of the study was to investigate the genotypic and phenotypic diversity of the species and to determine whether a correlation exists between genotype and ecological origin or spoilage potential, we wanted to constitute as diverse a strain collection as possible. First, 79 isolates from spoiled or non-spoiled meat, seafood and milk products were provided by different laboratories. We included DSM 20599 and the type strain ATCC 11509 (= DSM 20171), both isolated from pork meat. To complete the collection, chicken cuts and a beef slaughterhouse environment were sampled as no such isolate was represented in the available collection. New isolates from non-spoiled ground beef meat were

also added for comparison with those provided by other laboratories. More than 200 new isolates were collected and 80 were kept for further analysis after removal of putative redundant strains, using a preliminary rep-PCR analysis. In total, a collection of 161 *B. thermosphacta* strains was selected for analysis (Table 1).

3.2. *rpoB* species-specific PCR test

As *B. campestris* isolates are scarce (only 5 strains have been reported in the literature (Illikoud et al., in press) and references therein), we developed an accurate and reproducible PCR assay for a fast identification of *B. thermosphacta* isolates since both species cannot be discriminated through their 16S rDNA sequence. The PCR assay was designed to target a 394 bp region of the *B. thermosphacta rpoB* gene. The specificity of the primer set (*rpoB*-Fw1_154–175/*rpoB*-Rev1_525–547) was tested against a range of DNA from closely related bacterial species, such as *B. campestris*, and some *Listeria* or lactic acid bacteria species (Fig. 1A).

Amplification was only observed from *B. thermosphacta* DNA, showing the specificity of the assay. The 80 new isolates were then tested by this species-specific *rpoB*-PCR assay and all were identified as *B. thermosphacta*.

3.3. Characterization of genotypic the intra-species diversity

The diversity among the 161 *B. thermosphacta* strains was assessed by MALDI-TOF MS, rep-PCR, and PFGE typing methods. The *B. campestris* type strain ATCC 43754 was included as a control.

MALDI-TOF MS spectra were obtained for all strains. Cluster analysis was performed on all the spectra to visualize similarities between those of different strains. This generated a dendrogram composed of 14 groups, named from A to N (Supplementary Fig. S1). Two groups (M and N) encompassed about 87% of the collection (N = 113 and 26 strains, respectively) with other groups containing only 1 to 4 strains. Strains from different ecological origins were distributed in all groups. Most groups included strains isolated from different ecological origins while groups G, H, J, and L encompassed only 2–4 chicken cut isolates. *B. thermosphacta* TAP 107 and TAP 104 from group G came from the same sample, thus we cannot exclude that these two isolates are redundant. Conversely, *B. thermosphacta* TAP 204 and TAP 206 from group H were isolated from different batches. *B. campestris* ATCC 43754 was the only member of group B. By exploiting only the intra-species spectral variations, typing with MALDI-TOF did not report accuracy the phylogenetic distances between the two species of *Brochothrix* sp. For this reason MALDI-TOF MS did not clearly differentiate *B. thermosphacta* from *B. campestris* since another *B. thermosphacta* isolate (MFPA43A14-06) stayed as an outgroup (Supplementary Fig. S1).

The electrophoresis profiles of rep-PCR products yielded 3 to 11 bands, depending on strains. The amplification products were mainly in the range of 0.5–3 kb and a quite large diversity between profiles was observed. The UPGMA clustering analysis of the rep-PCR profiles obtained for all strains produced the dendrogram shown in Supplementary Fig. S2. *B. campestris* ATCC 43754 was clearly located in a separate external cluster whereas all *B. thermosphacta* strains formed a single cluster. By applying a 60% similarity coefficient, the dendrogram analysis generated 12 groups (named A to L) in the *B. thermosphacta* cluster. Each group was composed of at least two isolates from different ecological origins and different laboratory collections, except for group L, which included two isolates (TAP 105 and TAP 199) from different chicken cut batches. Some isolates, all from sea bream and the same collection (such as FMCC B-116, FMCC B-118, and FMCC B-119 from group I) had very similar profiles and may be redundant. This was again observed for TAP 107 and TAP 104 in group C. However, these two chicken meat isolates also harbored a profile very close to that of CRE 2333, EBP 3069, EBP 3070, SF 677, and SF 711, all from different seafood products and collected from different laboratories. Using a similarity coefficient of 80% was not more informative as it generated 50 groups, 20 of which were composed of a single strain.

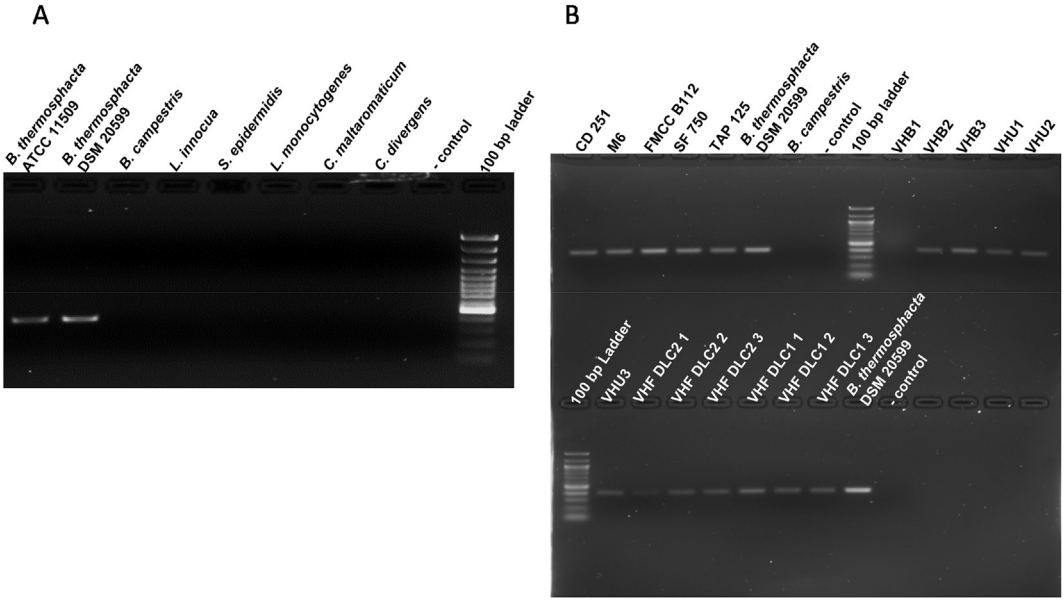

Fig. 1. Specificity of the specific PCR assay. A: Performance of the *B. thermosphacta*-specific PCR assay with DNA obtained from representative *Brochothrix* species and closely related bacteria. **B:** New isolates from chicken cuts (TAP 125) and beef meat (VHB2, VHB3, VHU1, VHU2, VHU3, VHF DLC2 1, VHF DLC2 2, VHF DLC2 3, VHF DLC1 1, VHF DLC1 2, and VHF DLC1 3) were identified as *B. thermosphacta*. Strains from external laboratories: *B. thermosphacta* ATCC 11509, *B. thermosphacta* DSM 20599, *B. campestris* ATCC 43754, CD 251, M6, FMCC B-112, and SF 750 were included. VHB1 isolate was negative and thus considered as not belonging to the *B. thermosphacta* species. A 100 bp ladder (New England Biolabs, France) and a negative (−) control were included.

By PFGE, using *Apa*I as the restriction enzyme, *B. thermosphacta* CD 355, MIP 2622, TAP 57, TAP 63 and TAP 78 showed only one band or could not be lysed. These isolates were therefore excluded from the analysis. The UPGMA cluster analysis of the PFGE profiles obtained for the remaining strains resulted in the dendrogram shown in Supplementary Fig. S3. The dendrogram analysis, applying a 35% similarity coefficient, generated 18 groups (A to R) with only 2 single-strain groups (*i.e.* D and R). Except for these two groups and group M composed of two strains isolated from cooked and peeled shrimp, each group comprised at least two strains from different ecological origins. In addition, these groups comprised strains from at least two different laboratory collections. With a 60% similarity coefficient, 75 groups were differentiated including 34 groups consisting of single strains. The dendrogram analysis with an 80% similarity coefficient generated 130 groups including 108 single-strain groups. PFGE did not differentiate *B. campestris* ATCC 43754 from *B. thermosphacta* strains. In fact, this strain appeared in group I with *B. thermosphacta* FMCC B-116, FMCC B-118, M1 and VHB2.

Multidimensional scaling (MDS), obtained by summing the two matrices of dissimilarities from the rep-PCR and PFGE analyses, produced a spatial configuration representing the distances between isolates (Fig. 2). It revealed significant intra-species diversity within the strain collection. MDS also clearly illustrated the absence of ecotype in *B. thermosphacta*: the intra-species diversity was not related to the ecological origin of the strains as isolates from the various origins were widely distributed and no strain clustering associated with a particular environment was observed.

3.4. Acetoin and diacetyl production

All strains were first screened to estimate their ability to produce acetoin in a laboratory medium using the Voges-Proskauer reaction. Levels of acetoin and diacetyl were estimated using a six-point scale, based on the color intensity, noted from (0): no production to (5): very high production. Negative (*E. coli* K12, which does not produce acetoin) and positive (acetoin-producing *S. liquefaciens* ATCC 27592) controls were included (Fig. 3A). The production level varied between strains. For example, 12% of the strains similar to the negative control and noted (0) did not produce acetoin, while only 2% produced very high levels (noted (5), far above the positive control) (Fig. 3B).

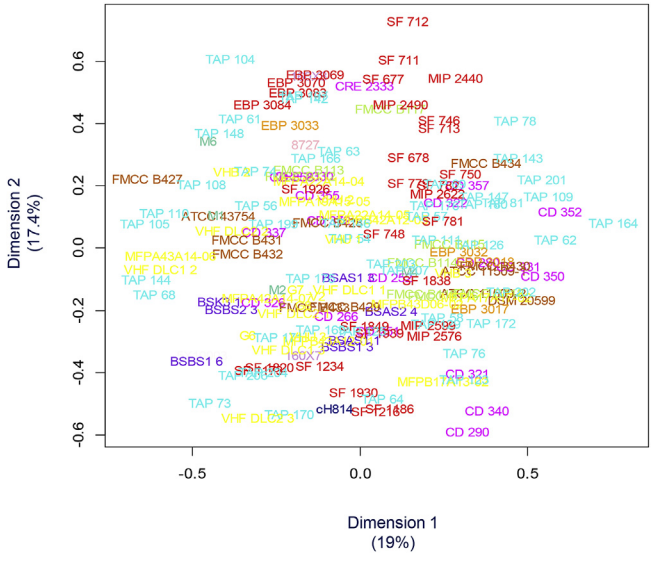

Fig. 2. Multidimensional scaling of summed rep-PCR and PFGE distance matrices. Each strain is presented with a color code illustrating its ecological origin (■): beef meat (■), pork meat (■), lamb meat (■), horse meat (■), beef + lamb meat (■), chicken meat (■), shrimp (■), salmon (■), sea bream (■), cod fillet (■), cheese, and (■) slaughterhouse environment.

Thirteen strains representing the 6 classes mentioned above (low to high producers) were chosen for further analysis to measure acetoin/diacetyl production after growth in meat and shrimp juices. Diverse strains were selected, taking into account the ecological and geographical origins and the diversity assessed by MALDI-TOF MS, rep-PCR and PFGE. The *B. thermosphacta* and *B. campestris* type strains and *B. thermosphacta* DSM 20599 were also included. All tested strains produced both molecules in meat and shrimp juices, and the production level was both strain- and food matrix-dependent (Fig. 4). For all strains, acetoin and diacetyl were produced at higher concentrations in meat juice than in shrimp juice. In meat juice, the production level ranged from 26.87 ± 8.56 μg/ml to 129.06 ± 41.32 μg/ml for diacetyl, and from 51.65 ± 6.32 μg/ml to 111.48 ± 2.84 μg/ml for acetoin. *B. thermosphacta* EBP 3070, a strain isolated from a spoiled fish product, was the highest producer in shrimp juice, with 22.86 ± 3.19 μg/ml acetoin and 30.24 ± 2.78 μg/ml diacetyl. In shrimp juice, the lowest production levels were observed with the *B. thermosphacta* type strain (5.30 ± 1.01 μg/ml acetoin and 5.41 ± 1.67 μg/ml diacetyl) and *B. thermosphacta* BSAS1 3 (7.05 ± 1.20 μg/ml acetoin and 7.94 ± 1.71 μg/ml diacetyl), which was isolated from the environment. *B. thermosphacta* EBP 3033, EBP 3070, and TAP 175 were among the higher producers of acetoin and diacetyl in shrimp juice and lower producers in meat juice. Conversely, CD 337 and BSAS1 3 were high producers of acetoin and diacetyl in meat juice and lower producers in shrimp juice. Finally, *B. campestris* ATCC 43754 was among the lowest acetoin and diacetyl producer, whatever the juice used. The quantitative data obtained on shrimp juice correlated with those of the screening test performed in BHI medium. Indeed, the highest producers EBP3033, EBP 3070, and TAP 175 (Fig. 4) were noted (5), (5), and (4), respectively using the six-point scale based color intensity of the preliminary test. On the opposite, the lowest producers ATCC 11509 and BSAS1 3 were noted (2) and (1), respectively.

Bacterial enumeration showed that the bacterial population reached at the end of the experiment was higher in shrimp juice than in meat juice. Counts varied according to strains from 6.06 ± 0.05 log CFU/ml to 6.72 ± 0.20 log CFU/ml in meat juice and from 7.75 ± 0.11 log CFU/ml to 8.31 ± 0.12 log CFU/ml in shrimp juice. After 48 h of incubation, the pH of non-inoculated meat and shrimp juices was 5.54 ± 0.19 and 6.40 ± 0.16, respectively. For all strains, the final pH reached 5.03 ± 0.062 and 5.66 ± 0.104 in meat and shrimp juices, respectively.

4. Discussion

The diversity of the microbial populations involved in the spoilage of meat and seafood products has been widely studied and documented (Dainty and Mackey, 1992; Jaffrès et al., 2011; Koutsoumanis and Nychas, 1999; Nychas et al., 2008; Remenant et al., 2015). These studies have shown that *B. thermosphacta* plays an important role in the spoilage of these products. Although the diversity of its spoilage potential depending on the strains has been described, it remains poorly understood (Casaburi et al., 2014). The aims of our study were to evaluate the intra-species diversity in a large and diverse collection of *B. thermosphacta* isolates and to investigate whether this diversity could be correlated with their ecological origin and/or their ability to produce spoilage compounds.

Half of the collection was constituted of new isolates collected for this study. Since the two closely related species *B. campestris* and

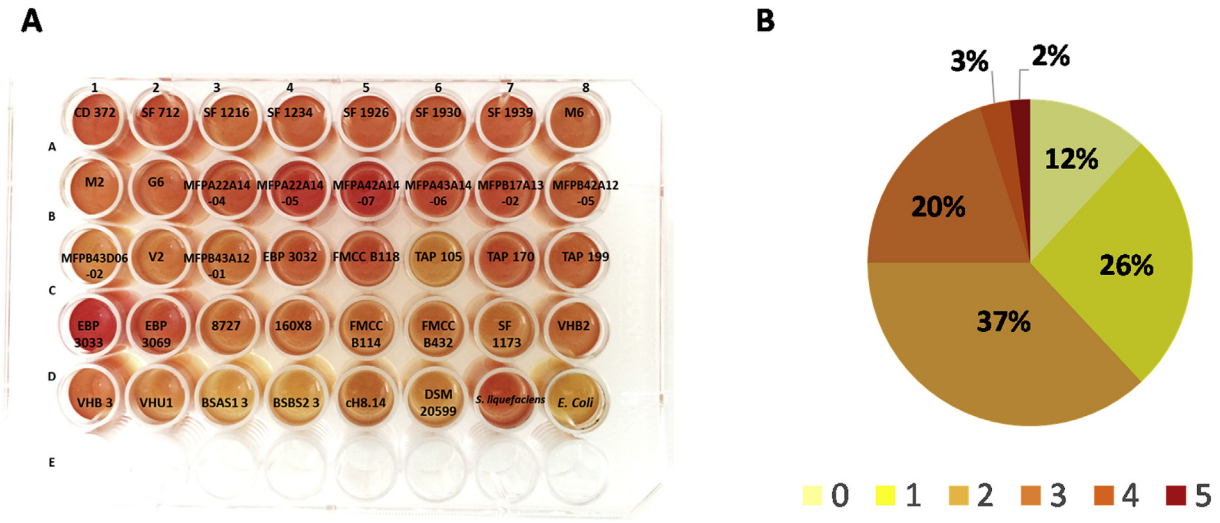

Fig. 3. Acetoin production using the Voges-Proskauer test. The level of acetoin production was visually estimated using a six-point scale based on the color intensity and noted from: (0): not produced (light yellow color) to (5): very high production (strong red color). **A:** Example of the results of the acetoin production assay for *B. thermosphacta* strains, *S. liquefaciens* (positive control), and *E. coli* (negative control). **B:** Pie-chart representing the overall results for 161 *B. thermosphacta* strains.

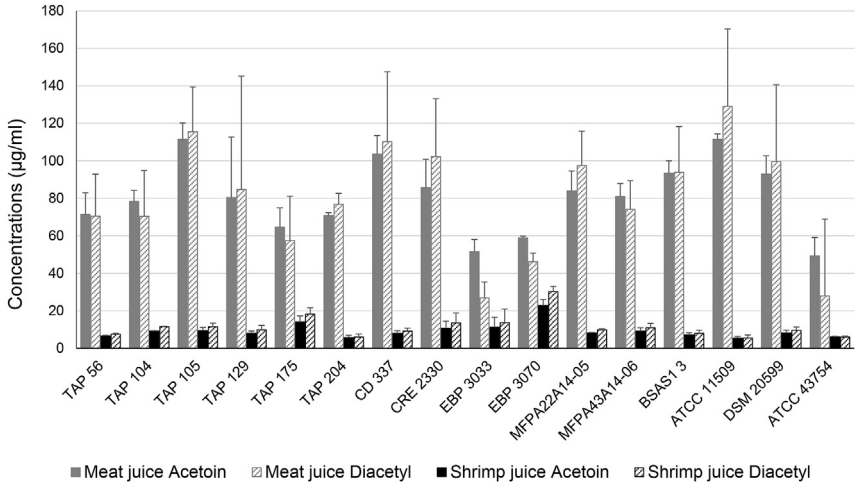

Fig. 4. Acetoin and diacetyl production by *Brochothrix* sp. strains in meat and shrimp juices. Fifteen *B. thermosphacta* strains and *B. campestris* ATCC 43754 were tested for their ability to produce acetoin and diacetyl after 48 h of culture in meat and shrimp juices. Data are expressed as the mean ± SD of three biological replicates.

B. thermosphacta cannot be distinguished through their 16S rDNA sequence, we developed a PCR assay based on the *rpoB* gene to identify *B. thermosphacta* accurately among the new isolates. The *rpoB* housekeeping gene, encoding the RNA polymerase beta-subunit, has been described as a useful and relevant target for bacterial identification and phylogenetic studies (Adékambi et al., 2009; Case et al., 2007; Mollet et al., 1997). The specificity of this PCR assay was confirmed against a range of DNA from closely related bacterial species, such as lactic acid bacteria and *Listeria* species. This enabled the differentiation of both *Brochothrix* species and the identification of new isolates as *B. thermosphacta*. In fact, of 207 chicken cut isolates, 206 were positive to the *rpoB* PCR test, and the other one was identified as *Enterococcus faecalis* by partial 16S rDNA sequencing. Similarly, 11 out of 12 ground beef new isolates were identified as *B. thermosphacta* by this PCR assay (Fig. 1B).

The collection included 161 *B. thermosphacta* isolates from the environment and various food matrices of animal origin (cheese; chicken, pork, beef, horse, and lamb meats; cod, salmon, sea bream, and shrimp). Some were isolated from spoiled food and others were collected from unspoiled products and before the use-by-date. MALDI-TOF MS, rep-PCR, and PFGE typing methods were selected to investigate the diversity in the collection because they are based on different principles. Rep-PCR provides fingerprints related to the presence of small repeats within the genome (Ishii and Sadowsky, 2009) while electrophoretic PFGE profiles are generated by the separation of DNA fragments after genomic DNA digestion with rare-cutting restriction enzymes (Li et al., 2009). MALDI-TOF generates peptide mass fingerprinting of proteins, mainly ribosomal ones as they are the most abundant and are constitutively synthesized (Rahi et al., 2016).

PFGE and rep-PCR revealed a significant diversity between *B. thermosphacta* isolates. Analysis of rep-PCR profiles by applying 60% or 80% similarity coefficients generated 12 and 50 groups, respectively. With PFGE dendrograms, these similarity coefficients distinguished 75 and 130 groups. Our results thus revealed a greater diversity than reported in previous studies on *B. thermosphacta*. For example, a PFGE analysis performed on 302 *B. thermosphacta* pork isolates distinguished only 8 groups

(Papadopoulou et al., 2012), while rep-PCR used on 27 *B. thermosphacta* isolates from meat, poultry and seafood reported only minor differences between isolates (Xu et al., 2010). The unique pork ecological origin in the first study and the small number of isolates in the second one most probably explain the low diversity reported by these authors. Compared to the observations made with the two DNA-based methods, MALDI-TOF MS typing showed a lower diversity as a major cluster encompassed more than 70% of the strains we tested. MALDI-TOF MS has been shown to discriminate *L. monocytogenes* (Barbuddhe et al., 2008; Ojima-Kato et al., 2016), *Lactobacillus brevis* (Kern et al., 2014), and *E. coli* (Siegrist et al., 2007) strains successfully. However, this method was not suitable for differentiating the two subspecies *Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* (Tanigawa et al., 2010). The same failure was reported for species belonging to the genera *Bacillus* and *Pseudomonas* (Ghyselinck et al., 2011), suggesting that the resolution of MALDI-TOF MS is taxon-dependent (Ghyselinck et al., 2011). To our knowledge, MALDI-TOF MS has been successfully used for identifying *B. thermosphacta* isolates from food (Höll et al., 2016), but never for investigating diversity within this species. Moreover, the available databases contain a limited number of spectra for some bacterial species.

Whatever the method used, we observed that the different ecological origins (meat, milk, seafood, or slaughterhouse environment) were widely distributed in all groups. In addition, most groups encompassing a reasonable number of strains (more than 2) included isolates from different ecological and geographical origins. Furthermore, most of the groups contained isolates from spoiled and non-spoiled food and from both processed and unprocessed products. This suggests that the strains belonging to the different groups may have a common contamination pattern in the various meat and seafood products. These strains have adapted to grow in chilled meat and seafood products. We also noted that the groups comprised isolates recovered from products of different meat animal species as well as from the processing environment. This probably reflects the physiological capability of *B. thermosphacta* strains to grow in various food matrix ecosystems independently of the product type (raw or processed), the animal species from which it is derived (beef, pork, salmon, shrimp, etc.), and the packaging conditions (under air, vacuum packaged, modified atmosphere). In other words, no ecotype (strains sharing the same ecological niche) was observed. Of the 80 new isolates, for which information about batch origin was available, a few clustered together systematically, whatever the typing method used. Five pairs of such isolates (TAP 57/TAP 63, TAP 104/TAP 107, TAP 143/TAP 147, TAP 176/TAP 180, and TAP 204/TAP 206) were noticed and were collected from the same batches. Nevertheless, TAP 104, TAP 107, TAP 176, TAP 180, TAP 204, and TAP 206 showed differing acetoin production ability in the Voges-Proskauer test and were therefore not considered redundant.

Whether the spoiling potential of *B. thermosphacta* is strain-dependent is unknown. To evaluate this, we focused on acetoin and diacetyl production since both molecules have already been reported as associated with the spoilage of beef and chicken meat and seafood products (Casaburi et al., 2014; Franke and Beauchamp, 2017; Jaffrès et al., 2011). We first screened the ability to produce acetoin using the Voges-Proskauer reaction from glucose fermentation in BHI laboratory medium. Our results showed that the production of this molecule was highly variable between strains and did not correlate with their origin (spoiled or non-spoiled products, nature of the food) or their genotypic clustering.

The combination of rep-PCR, MALDI-TOF and PFGE clustering and Voges-Proskauer reaction data was used to select a sub-set of thirteen distant strains for acetoin and diacetyl quantification. For this, sterile juices from two food matrices, beef meat and shrimp, were used in this study in order to avoid interference with the endogenous microbiota. Both acetoin and diacetyl were produced by *B. thermosphacta* in both matrices. These results differ from previous studies that reported no acetoin but only diacetyl production in cooked and peeled shrimp packed under modified atmosphere (Jaffrès et al., 2011; Laursen et al., 2006). Conversely, Casaburi et al. (2014) reported that *B. thermosphacta* produced acetoin but not diacetyl in beef meat. These apparent contradictions may result from different methods and experimental conditions (shrimp packed under modified atmosphere *vs.* shrimp juice; pieces of beef meat stored aerobically *vs.* beef juice). Acetoin and diacetyl production was higher in meat juice than in shrimp juice although the bacterial population reached after 48 h of incubation was higher in shrimp juice than in meat juice. These observations could suggest that the production of these molecules is not related to the growth level of *B. thermosphacta*, but more probably to the biochemical composition of the food matrix. Moreover, acetoin and diacetyl production levels varied between strains. We noticed that *B. thermosphacta* EBP 3070 (isolated from spoiled salmon) and TAP 175 (isolated from non-spoiled chicken cuts) were among the highest producers of acetoin and diacetyl in shrimp juice but among the lowest ones in meat juice. Conversely, *B. thermosphacta* CD 337 (isolated from spoiled shrimp) and BSAS1 3 (isolated from the slaughterhouse environment) belonged to the highest acetoin and diacetyl producers in meat juice and to the lowest in shrimp juice. This shows that the spoilage ability of *B. thermosphacta* was both strain- and matrix-dependent but was not correlated to the food from which environment strains were isolated. This might result from the regulation of genes involved in the metabolic pathways that produce these molecules, which may vary depending on strains. This was recently suggested by the comparison of 13 *B. thermosphacta* draft genomes, which highlighted a large number of transcriptional regulators in these genomes but a small difference between strains (Stanborough et al., 2017). However, the genomes were all sequenced from strains of meat origin and may not represent the diversity of the *B. thermosphacta* species.

5. Conclusion

The present study revealed a significant diversity within the strain collection using rep-PCR, PFGE and MALDI-TOF typing methods. All these methods showed that there was no ecotype in this *B. thermosphacta* strain collection. The ability to produce acetoin and diacetyl in meat and shrimp juices varied between strains and did not correlate with the isolation from a spoiled or non-spoiled food product, suggesting that the spoiling ability of *B. thermosphacta* was most probably linked to strain properties rather than to the food environment from which they were isolated. Based on these results four strains were selected for genome sequence comparison, and transcriptomics coupled to volatilome analysis will be performed for better understanding of the *B. thermosphacta* spoilage mechanisms.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.01.015.

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