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Activities of strains of *Brochothrix thermosphacta in vitro* and in meat

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ABSTRACT

Ninety three isolates of *Brochothrix* spp. from raw meat were analysed by PCR-based molecular fingerprinting and identified by species-specific Real-Time (RTi)-PCR and 16S rRNA gene sequencing. Thirty three strains were characterized for their growth capability in sarcoplasmic extract with and without glucose, for *in vitro* lipolytic activity, *in vitro* and *in situ* (in beef) proteolytic activity and for amino-decarboxylase activity. Moreover, spoilage potential of seven selected strains in sterile and non-sterile meat was investigated by analyzing the release of volatile organic compounds (VOCs) during storage in air. All the strains analysed were unable to hydrolyze sarcoplasmic proteins *in vitro* and *in situ* and they did not show lipolytic activity at 4 °C or 20 °C. Almost all were able to grow in the presence of sarcoplasmic extract with glucose and produced histamine. The release of VOCs by each strain in sterile and non sterile beef stored at 4 °C in air was evaluated by HS-SPME-GC/MS analysis. Acetoin and 1-octen-3-ol and 3-methyl-1-butanol were the major compounds isolated from sterile and non-sterile meat samples inoculated with single strains of *Brochothrix (B.) thermosphacta* and high concentration of acetoin was found in all inoculated meat samples. The role of *B. thermosphacta* as meat spoiler does not seem to be influenced by indigenous microbiota of meat while its development in meat is associated to a significant increase ($P < 0.05$) of acetoin and other compounds recognized as important contributors to the spoilage of meat and meat products.

1. Introduction

Microbial spoilage of meat is a complex event to which many different bacterial populations can contribute depending on the temperature of storage and packaging conditions. It is the most common cause of deterioration and may appear as visible growth (slime, colonies), as textural changes, off-odours or off-flavours. The spoilage potential of a microorganism is determined by its ability to produce the metabolites that are associated with the spoilage. However, it is also important to consider the interaction between microbial growth and enzyme activities (Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). Several groups of organisms contain members potentially contributing to meat spoilage under certain conditions (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2014; Doulgeraki, Ercolini, Nychas, & Villani, 2012) and only continuous and updated knowledge on the biological and molecular diversity of species and strains involved in the spoilage of the meat can allow a complete understanding of spoilage.

The effect of *Brochothrix thermosphacta* strains on the overall quality of meat and meat products can be very important. It is noteworthy that according to Regulation 178/2002 of the European Parliament and Commission, which sets forth the general legal provisions related to foods, a foodstuff is regarded as unsafe not only if it is harmful to consumer health, but also if it is not fit for human consumption. The genus *Brochothrix* includes two species, *B. thermosphacta* and *Brochothrix campestris*. *Brochothrix* organisms are Gram-positive, non spore forming, non motile, homofermentative, facultative anaerobic rod-bacteria occurring singly, in short chains or long filamentous chains (Stackebrandt & Jones, 2006). *B. thermosphacta* was first isolated from pork and currently the information available suggests that *Brochothrix* spp. are widely distributed in the environment and become a dominant part of the microbiota in habitats that selectively favour their growth. It has been found in meat, seafood, as well as tools used in meat processing (Nychas et al., 2008; Papadopoulou, Doulgeraki, Botta, Cocolin, & Nychas, 2012). It appears to be among the dominant bacteria associated with the spoilage of refrigerated meat along with *Pseudomonas*, lactic acid bacteria, and Enterobacteriaceae (Casaburi et al., 2011; Doulgeraki et al., 2012) and it may become the dominant spoilage species in chilled meat when oxygen is present (Gribble & Brightwell, 2013). Its capability to grow on meat in both air storage and vacuum storage make it a significant meat colonizer and an important player also in meat stored under vacuum (Kakouri & Nychas, 1994; Sheridan et al., 1997). *B. thermosphacta* displays variable biochemical properties that change its pattern of metabolism under different growth conditions. In fact, it is able to ferment not only glucose but also other sugars such as ribose,

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fructose and maltose, showing the ability to activate various metabolic pathways for sugar breakdown (Samelis, 2006). Gill and Newton (1977) suggested that glucose was the substrate preferentially used by one strain of *B. thermosphacta* when growing in meat. In fact, no utilization of glucose-6-phosphate, lactic acid and nucleotides was detected and the range of amino acids that this bacterium could use was very narrow. Under anaerobic conditions, the main metabolites resulting from consumption of glucose by *B. thermosphacta* are L-(+)-lactic acid and ethanol, but no acetoin and only small or no amounts of short chain fatty acids have been detected (Dainty, Shaw, Harding, & Michanie, 1979). In aerobic conditions, a strain of *B. thermosphacta* was found to produce acetoin, acetic, isobutyric, 2-methylbutyric and isovaleric acids and 3-methylbutanol (Pin, Garcìa de Fernando, & Ordòñez, 2002). Stanley, Shaw, & Egan (1981) depicted *B. thermosphacta* as a bacterium with considerable significance in spoilage of meat describing the spoilage after the first days of storage as an odour rather than a flavour defect. The off-odour became significantly different from the odour of uninoculated meat samples when the bacterial population had reached 10^8 CFU/g.

The nature of quality deterioration associated with *B. thermosphacta* seems to be principally due to off-flavours, discoloration and gas production (Braun & Sutherland, 2003), and pungent 'cheesy' odour (McClure, Baranyi, Boogard, Kelly, & Roberts, 1993). There is scarce information on the behaviour of different strains belonging to *B. thermosphacta* during their growth in meat and about their capability to produce specific volatile compounds. The studies available in literature report the ability of few strains of *B. thermosphacta* to grow in laboratory media or meat surface (Russo, Ercolini, Mauriello & Villani 2006; Dainty, Edwards, & Hibbard 1989; Gill & Newton, 1977) and the ability of some strains to produce end-products in laboratory media and in naturally spoiled meat (Dainty & Hofman 1983; Ercolini et al., 2011). The aim of this study was to identify and characterize *B. thermosphacta* isolates from meat, and to investigate the spoilage potential of the strains by measuring the production of volatile organic compounds (VOCs) in air-stored sterile and non sterile beef.

2. Material and methods

2.1. Isolation of bacteria

Ten 500 g-portions of beef (*Longissimus dorsi*), designated from B to M, were obtained from local butchers and after storage in air or vacuum packages for 0, 7 and 20 days (Pennacchia, Ercolini, & Villani, 2011) were used as source of isolation of *B. thermosphacta* on STAA agar base (Oxoid, Garbagnate Milanese, Italy) with STAA selective supplement (Oxoid) incubated at 22 °C for 48 h. Five colonies were isolated from each sample and a collection of 250 isolates was obtained. Purified isolates were characterized by microscopic observations, Gram and catalase reactions. Ninety-three isolates resulted Gram positive and catalase positive and were characterized in this study. The isolates were routinely grown in Tryptone Soya Broth (TSB Oxoid) with 0.5% yeast extract (Oxoid) after an aerobic incubation for 24–48 h and the working cultures were maintained in TSB with 25% glycerol at −20 °C.

2.2. Molecular typing by RAPD-PCR and cluster analysis

DNA was extracted using the Wizard DNA purification kit (Promega, Madison, WI) following the manufacturer's instructions. After DNA precipitation with 0.7 vol. of isopropanol, the resulting pellet was washed with 70% ethanol. Finally, 5 μl of 10× RNAse buffer and 0.5 μl of RNAse (Promega) were added and the solution was incubated at 37 °C for 30 min. The DNA extracted was stored at −20 °C.

For the molecular typing of the 93 isolates, Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) and repetitive element sequence based-PCR (rep-PCR) were used with primers XD5 (5′-CTGGCGGCTG-3′) (Ercolini, Russo, Nasi, Ferranti, & Villani, 2009) and GTG_5: (5′-GTG GTG GTG GTG GTG-3′) (Gevers, Huys, & Swings, 2001), respectively. PCR reactions were carried out in 25 μl of reaction mix with 20 ng of the template DNA as previously described (Ercolini et al., 2009). A database of fingerprints was created by using the software Bionumerics version 5.1 (Applied Maths, St. Martens Latem, Belgium). A combined data matrix of all the fingerprints obtained using two different RAPD-PCR conditions was obtained and a similarity dendrogram was created by using the Dice coefficient and the unweighted pair group method using an arithmetic mean (UPGMA) clustering algorithm (Vauterin & Vauterin, 1992).

2.3. Identification of B. thermosphacta by Real-Time-PCR and 16S rRNA gene sequencing

The primers Bcr3r (5′-GTT GTC CGG AAT TAT TGG G-3′) and Bcr3f (5′-CTC CTC TTC TGT CCT CAA G-3′) described by Pennacchia, Ercolini & Villani (2009) ere used in this study to identify strains of *B. thermosphacta* by Real-Time (RTi)-PCR. The expected size of the PCR product was 121 bp. The amplification reactions were performed in a total volume of 25 μl and every strain was processed in triplicate. The experiments were performed in the Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories, Milan, Italy) as previously described (Pennacchia, Ercolini, & Villani 2009).

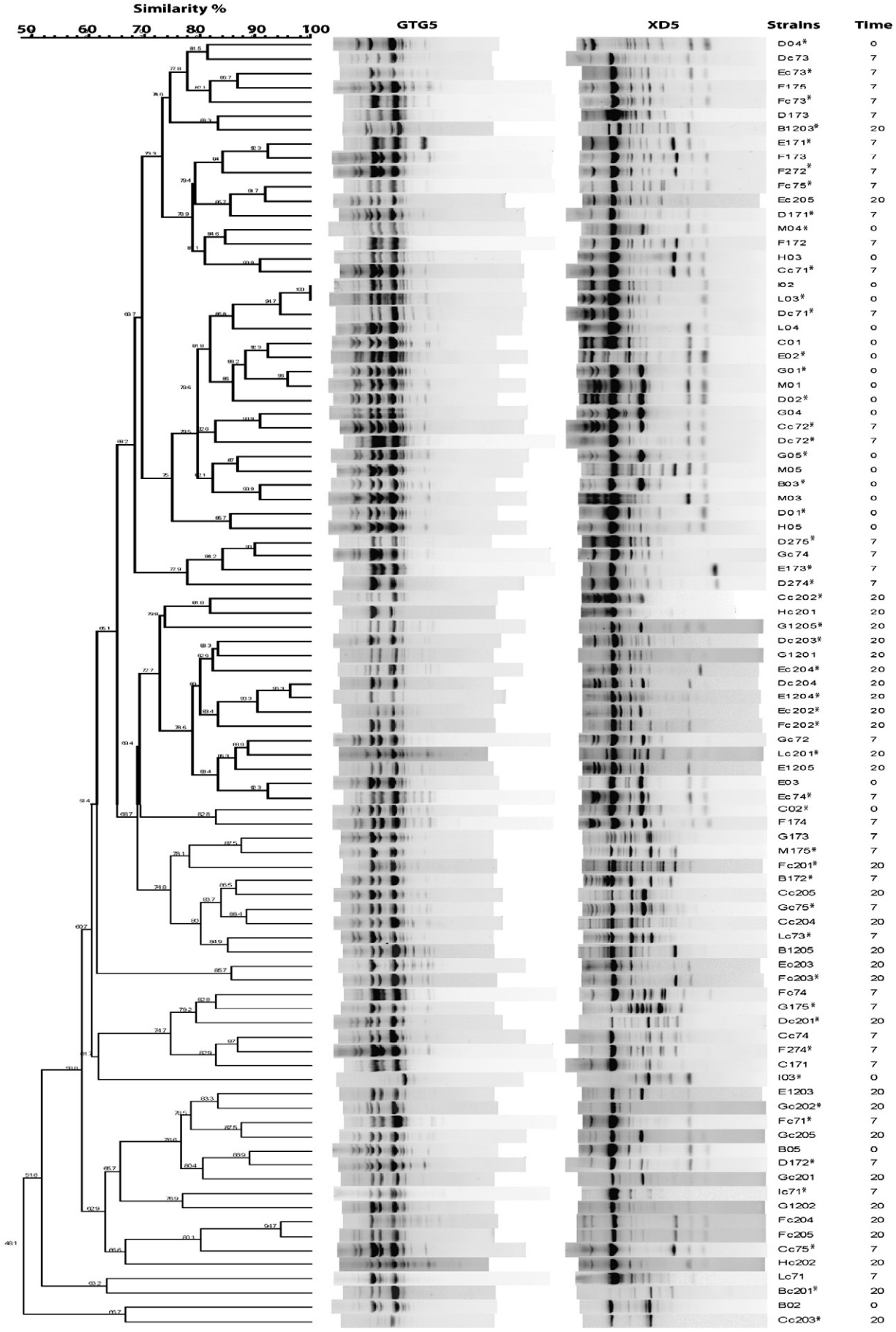
Representative isolates, chosen on the basis of the molecular typing by RAPD-PCR, were identified by 16S rRNA gene sequencing. In order to amplify the 16S rRNA gene, the oligonucleotide primers were used, described by Weisburg, Barns, Pelletier, & Lane (1991). The PCR mixture and conditions were as previously reported (Ercolini et al., 2009). PCR products from the 16S rRNA gene (1.6 kb) were purified by the QIAquick PCR Purification Kit (Qiagen) according to the supplier's instructions. The DNA sequence was determined by the dideoxy chain termination method by using the primer pair described above (Weisburg et al., 1991). DNA sequence alignments were performed using the National Centre of Biotechnology Information GeneBank (NCBI) online tools (Altschul et al., 1997).

2.4. Growth in sarcoplasmic extract

Thirty-three strains selected as representative of clusters obtained by RAPD-PCR fingerprints with similarity lower than 80% were used in order to evaluate their ability to grow in sarcoplasmic extract with and without glucose. Beef sarcoplasmic proteins were extracted as described by Mauriello, Casaburi, & Villani (2002). The strains (OD_{nm} of 0.1) were inoculated in triplicate in 0.2 ml of sarcoplasmic extract with and without 0.1% of glucose. Growth curves were obtained at 4 °C for 360 h by using the 96 well Bio-Tek Elx808 microtiter plate reader at 600 nm (Bio-Tek Instruments, Inc. VT, USA) with readings performed every 5 h. Spectrophotometric data were processed by a modified Gompertz equation (Zwietering, Jongerburger, Rombouts, & Van 't Riet, 1990) in order to calculate the growth curve parameters, *i.e.* lag time, maximum growth rate (μ_{max}) and maximum population level. Results were calculated as the means of three determinations. Non inoculated control samples were included in the analysis and blanked data were used for the modelling. The data of three repeated trials were analysed by *t*-test (Systat software, v.5.2.1 for Mac) in order to verify the differences between the strains during growth.

2.5. Proteolytic and lipolytic activity

The proteolytic activity of the 33 *B. thermosphacta* strains was determined *in vitro* and in sterile meat samples. For *in vitro* proteolytic activity, overnight cultures of the different strains were centrifuged, the pellets resuspended in 20 mM phosphate buffer pH 7.0 and inoculated (about 10^4 CFU ml^{-1}) in 10 ml of sarcoplasmic extract supplemented with 0.1% of glucose. Uninoculated sarcoplasmic extract was used as control and incubated under the same conditions at 4 °C. For


Similarity %
50
60
70
80
90
100
GTG5
XD5
Strains
Time
D04* 0
Dc73 7
Ec73* 7
F175 7
Fc73* 7
D173 7
B1203* 20
E171* 7
F173 7
F272* 7
Fc75* 7
Ec205 20
D171* 7
M04* 0
F172 7
H03 0
Cc71* 7
I02 0
L03* 0
Dc71* 7
L04 0
C01 0
E02* 0
G01* 0
M01 0
D02* 0
G04 0
Cc72* 7
Dc72* 7
G05* 0
M05 0
B03* 0
M03 0
D01* 0
H05 0
D275* 7
Gc74 7
E173* 7
D274* 7
Cc202* 20
Hc201 20
G1205* 20
Dc203* 20
G1201 20
Ec204* 20
Dc204 20
E1204* 20
Ec202* 20
Fc202* 20
Gc72 7
Lc201* 20
E1205 20
E03 0
Ec74* 7
C02* 0
F174 7
G173 7
M175* 7
Fc201* 20
B172* 7
Cc205 20
Gc75* 7
Cc204 20
Lc73* 7
B1205 20
Ec203 20
Fc203* 20
Fc74 7
G175* 7
Dc201* 20
Cc74 7
F274* 7
C171 7
I03* 0
E1203 20
Gc202* 20
Fc71* 7
Gc205 20
B05 0
D172* 7
Gc201 20
Ic71* 7
G1202 20
Fc204 20
Fc205 20
Cc75* 7
Hc202 20
Lc71 7
Bc201* 20
B02 0
Cc203* 20

the determination of proteolytic activity, beef muscles (*L. dorsi*) were superficially decontaminated as previously described (Ercolini et al., 2009) and decontaminated meat chops (about 40 g) were spiked with a suspension in quarter strength Ringer's solution (Oxoid) of each of the above strains to reach a final concentration of 10^3–10^4 CFU g^{-1}. The microbial load of the inoculated meat was routinely checked to assure that the desired inoculum was always reached. The inoculated and uninoculated meat samples and sarcoplasmic extracts were analysed by SDS-PAGE for protein hydrolysis after 0, 7 and 12 days of incubation at 4 °C (Casaburi et al., 2011). *B. thermosphacta* strains were enumerated on STAA agar in all the samples after 0, 7 and 12 days of incubation at 4 °C.

Lipolytic activity was tested on Spirit Blue Agar plates supplemented with a mixture of olive oil and Tween 80 according to the supplier's instructions (Sigma, Milan, Italy); *Staphylococcus aureus* ATCC25923 was used as positive control. Duplicate plates were inoculated with streaks of the *B. thermosphacta* strains and incubated for 3 days at 20 °C and 4 °C. After the incubation, lipolytic activity was detected by the formation of a clear zone around the colony (Immanuel, Palanichamy, Jebadhas, Iyapparaj, & Palavesam, 2008).

2.6. Amino-decarboxylase activity

The production of biogenic amines was evaluated in Decarboxylase broth (Dec-broth; pH 6.8; Falkow, 1958). The broth was prepared as described by De Filippis et al. (2013) using thiamine, hystidine, lysine and ornithine as amino acids. 10 ml of Dec-broth were inoculated with 1 ml of an overnight culture of each strain and incubated at 4 °C. Each strain was inoculated also into 10 ml of Dec-broth without amino acids (broth control) and incubated in the same conditions. A color change was checked every 24 h, comparing the Dec-broths inoculate with control inoculate Dec-broths. The production of amines raises the pH of the medium above 6.0, changing the color of the indicator from yellow to purple or violet.

2.7. Meat contamination by B. thermosphacta and storage in aerobic conditions

To investigate the spoilage potential in sterile and non-sterile meat, seven strains of *B. thermosphacta* were selected considering the maximum population level reached in sarcoplasmic extract with 0.1% of glucose. In particular, four strains of *B. thermosphacta* with $OD_{nm} > 0.6$ and three strains with $OD_{nm} < 0.6$ after 360 h at 4 °C were selected. Decontaminated or not decontaminated meat chops (about 40 g) were inoculated as described above and non-inoculated meat chops were included as controls. The strains were inoculated singly and the experiments were performed in duplicate (the same strain inoculated in two different pieces of meat, stored in identical conditions and analysed separately). The decontaminated or not decontaminated meat was placed in glass bottles (500 ml) and incubated at 4 °C for 7 days (Ercolini et al., 2010). After 0 and 7 days of aerobic storage, viable counts were performed on STAA and volatile organic compounds (VOCs) were determined by GC/MS as described below.

2.8. Volatile organic compounds' (VOCs) determination by GC/MS

VOCs determination was performed on contaminated sterile and non-sterile meat after 0 and 7 days of aerobic storage at 4 °C. The contaminated meat was placed in glass bottles (500 ml) that were closed and incubated at 4 °C for 7 days. The bottle size was chosen in preliminary trials because it allowed a suitable quantity of O_2 to be left at the end of incubation in order to assume that the growth had occurred aerobically (Ercolini et al., 2010). The SPME (Solid Phase Micro Extraction) analysis was carried in the headspace of the hermetically closed bottle by exposing the fiber (CAR/DVB/PDMS; Supelco Sigma-Aldrich, Bornem, Belgium) for 1 h. The HS-SPME-GC/MS analysis of meat samples was performed using equipment and conditions previously described (Casaburi et al., 2011). The masses were scanned on m/z range of 45–350 amu. For the identification of volatile components the NIST library and comparison with spectra and retention times of standards were used. The compounds identified with the use of standards were: acetoin, 2-heptanone, 3-octanone, hexanal, heptanal, nonanal, 1-hexanol, 3-methyl-1-butanol, 2-butoxyethanol, heptanol, 1-octen-3-ol, 1-octanol, 2-octen-1-ol and 2-ethyl-1-hexanol purchased from Sigma-Aldrich (St Louis, Mo, United States). The remaining compounds were identified by library comparison of the mass spectra. Calibration was performed by spiking sterile meat samples with γ-butyrolactone (2 μg for all samples) and with the standards listed above at 5 concentration levels in ranges where goodness-of-fit of linear regression was evaluated ($r^2 \geq 0.95$). Non inoculated control samples were included in the assays and blanked data were used for the analysis. The data of the three repeated trials were tested by analysis of variance using SPSS (13.0 Windows) in order to evaluate the differences between the strains. Statistical significance was identified at the 95% confidence level ($P \leq 0.05$).

3. Results

3.1. Identification of B. thermosphacta from meat and molecular typing

After purification and preliminary characterization, ninety-three Gram positive and catalase positive bacterial isolates were identified by Real-Time PCR, they were all assigned to *B. thermosphacta* and further analysed by RAPD-PCR. Furthermore, fifty strain representatives of different groups were confirmed to be *B. thermosphacta* according to 16S rRNA gene sequencing (99% closest relative accession number AY543029). The fingerprints and the dendrogram from the molecular typing are shown in Fig. 1. A considerable part of the strains resulted to be grouped in clusters with a high level of similarity of about 80%. Thirty-three strains belonging to different sub-clusters with similarity between 73% and 66% were selected for further characterization.

3.2. Growth in sarcoplasmic extract with and without glucose

The 33 strains of *B. thermosphacta* were characterized by the analysis of growth curves in sarcoplasmic extract with and without 0.1% of glucose at 4 °C. Spectrophotometric data were processed by a modified Gompertz equation to calculate lag time, μ_{max} and maximum population level. All the strains analysed showed low growth ability in the sarcoplasmic extract with mean values of maximum population of about 10^6 CFU/g (Fig. 2a). The addition of glucose determined a significant variation ($P \leq 0.05$) of all growth parameters except for the lag time (data not shown). In the presence of glucose the maximum population level increased significantly ($P \leq 0.001$) for all the strains analysed (Fig. 2a) while the μ_{max} values increased only for about 55% of strains (Fig. 2b). The highest population levels were reached in the presence of glucose by the strains M175, D01 and G01 (Fig. 2a).

3.3. Amino-decarboxylase, proteolytic and lipolytic activities

All the strains of *B. thermosphacta* tested were able to produce histamine and none produced other biogenic amines such as tyramine, cadaverine and putrescine. None of the strains analysed showed

Fig. 1. Dendrogram of similarity generated by RAPD-PCR fingerprints. A combined data matrix of all the fingerprints was defined and the dendrogram of similarity was obtained by using the unweighted pair group method using arithmetic average (UPGMA) clustering algorithm Vauterin & Vauterin (1992). Strain codes indicate: the meat sample used for isolation (B to M), aerobic (c) and vacuum (1) storage conditions and time of isolation (0, beef at time zero; 7, after 7 days and 20, after 20 days of storage at 4 °C). Codes labelled with asterisks indicate strains that were actually identified by 16S rRNA gene sequencing.

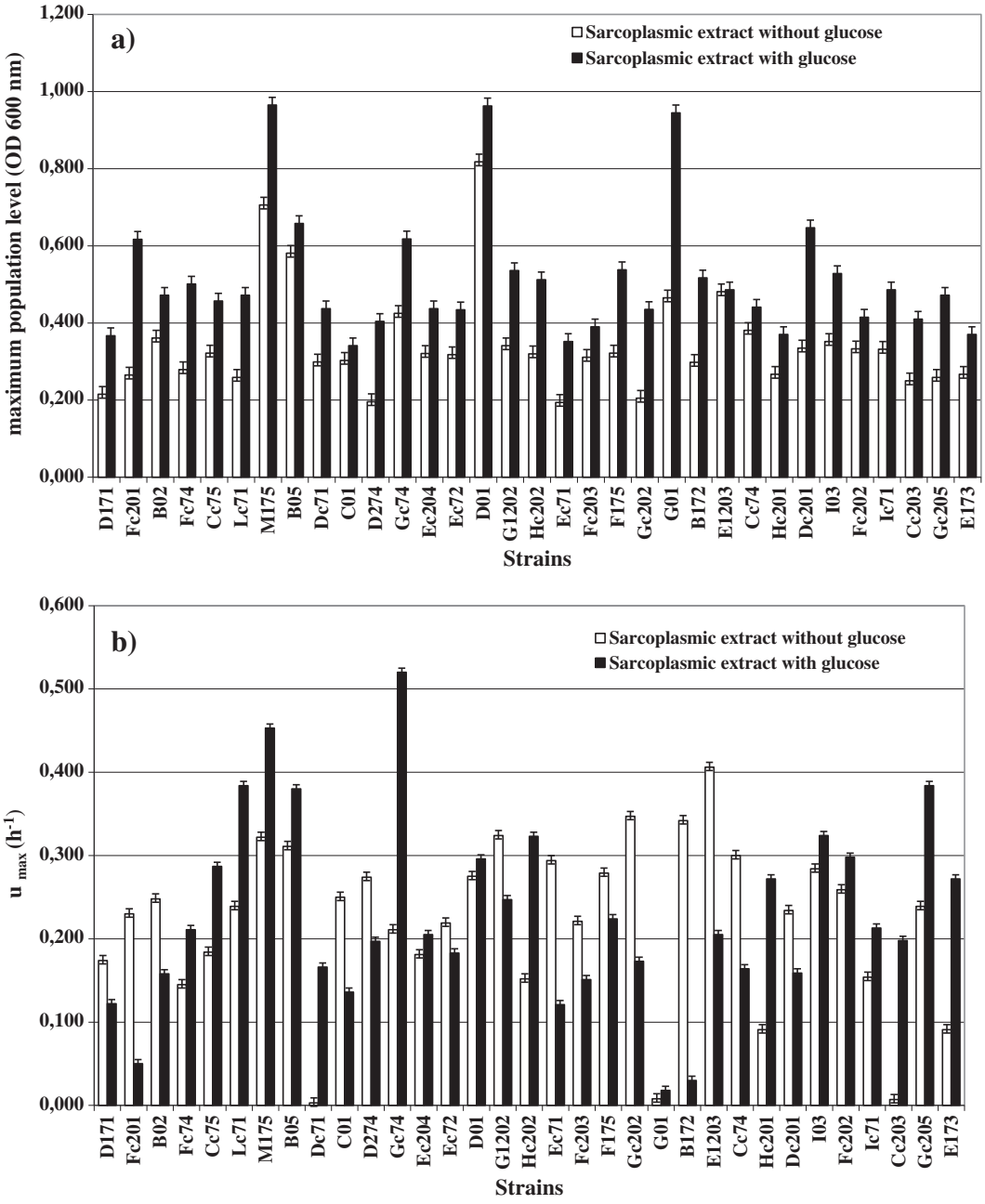


Fig. 2. Growth behaviour of *B. thermosphacta* strains in terms of (a) maximum population level (O.D. 600 nm) and (b) μmax (h^{-1}) in sarcoplasmic extract with and without glucose. The number of strains tested was 33, the tests were performed in triplicate.

lipolytic activity at 4 °C and 20 °C. After 12 days of incubation at 4 °C in sarcoplasmic broth, the protein profiles of uninoculated control, as well as those inoculated with the 33 single strains of *B. thermosphacta* were unchanged (data not shown). Although the viable counts of inoculated sterile meat samples on TSA agar after 7 and 12 days at 4 °C in air increased to 10^6–10^8 CFU g^{-1} and 10^8–10^{10} CFU g^{-1}, respectively, no changes were observed in sarcoplasmic protein profiles after growth of the strains in sterile meat. Similar results were obtained from the uninoculated sterile meat showing the absence of activity from endogenous muscle proteinases.

3.4. Production of volatile organic compounds (VOCs) in sterile and non-sterile beef

The initial viable count of inoculated sterile and non-sterile meat samples on STAA agar was about 10^3–10^5 CFU g^{-1}. After 1 week of incubation at 4 °C in aerobic conditions the load increased to 10^5–10^8 CFU g^{-1} in non-sterile meat samples and 10^6–10^8 in sterile meat samples (Table 1).

After GC/MS analysis, 38 VOCs were identified in the headspace (HS) of meat samples inoculated and uninoculated with the seven *B. thermosphacta* strains. The volatile fraction of meat samples included ketones, alcohols, aldehydes, some esters and two carboxylic acids (Table 2). Aldehydes and carboxylic acids were detected in traces in some inoculated meat. In particular, 3-methylbutanoic 'and 2-methylbutanoic acids were detected in sterile meat inoculated with the strains Cc75 and Dc201 and in non-sterile meat inoculated with the strain Gc202. The production of esters was overall greater in uninoculated sterile and non-sterile meat, except for non-sterile samples inoculated with the strain Dc71 ($P < 0.05$, Table 2). The quantity measurements of VOCs in beef samples uninoculated and inoculated with seven strains of *B. thermosphacta* are reported in Table 3. Ketons

Table 1
Viable counts of strains of *B. thermosphacta* on STAA agar in inoculated and uninoculated (negative control) sterile and non-sterile meat samples after 0 and 7 days of storage at 4 °C in aerobic conditions.

Control and strain inoculated samples*	CFU g^{-1}			
	Sterile (S)		Non-sterile (NS)	
	0	7	0	7
Control	<10	5.0×10^2	3.0×10^3	8.2×10^6
Cc75	3.7×10^4	1.7×10^8	2.2×10^5	3.6×10^8
Control	1.5×10	4.0×10^3	4.5×10	3.1×10^7
D01	1.4×10^3	1.5×10^5	3.0×10^3	5.4×10^7
Control	2.0×10	1.0×10^2	1.5×10^4	6.2×10^5
Gc202	2.6×10^3	5.2×10^6	2.5×10^3	2.0×10^6
Control	<10	1.9×10^3	3.6×10	1.0×10^5
Gc74	1.0×10^3	5.0×10^6	1.0×10^3	2.6×10^6
Control	<10	5.0×10^2	3.0×10^3	8.2×10^6
M175	2.0×10^3	5.0×10^6	1.0×10^3	2.1×10^6
Control	<10	1.7×10^4	1.0×10^2	1.0×10^6
Dc201	2.3×10^4	5.0×10^8	2.0×10^4	2.2×10^8
Control	1.2×10	2.5×10^4	1.0×10^3	1.0×10^7
Dc71	1.7×103	1.3×10^8	1.3×10^3	1.3×10^8

* The values are expressed as means based on duplicate experiments. Standard deviations were always lower than 20% of the means.

Table 2
Compounds detected in the headspace of sterile (S) and non-sterile (NS) meat samples uninoculated and inoculated with 7 strains of *B. thermosphacta* after 7 days of storage at 4 °C in aerobic conditions.

	Control NS	Control S	Dc71 NS	Dc71 S	Dc201 NS	Dc201 S	M175 NS	M175 S	Cc75 NS	Cc75 S	D01 NS	D01 S	Gc74 NS	Gc74 S	Gc202 NS	Gc202 S
Ketones																
Acetoin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2-eptanone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-octanone	+	+	+	+	+	+	+	+	+	+			+	+	+	+
2-nonanone			+	+	+											
Alcohols																
2,3-butandiol					+					+						
3-methyl-1-butanol	+	+	+	+	+	+	+	+			+	+	+	+	+	+
1-hexanol	+	+		+	+	+	+	+					+	+		+
2-ethyl-1-hexanol	+	+	+		+		+	+		+			+	+	+	+
1-heptanol	+	+			+	+	+	+			+		+	+	+	+
1-octanol	+					+	+	+			+		+	+	+	+
1-octen-3-ol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2-ethyl1decanol			+	+	+	+	+	+					+	+	+	
2-butoxyethanol	+	+	+	+	+	+	+						+		+	
Aldehydes																
Hexanal		+						+								+
Heptanal																+
Nonanal							+	+								+
Esters																
Ethylbutanoate	+	+	+	+	+	+	+	+			+	+	+	+	+	
3-methylethylbutanoate	+		+	+	+	+			+	+					+	+
Ethy-2-butanoate	+		+													
Ethyl-2-methyl, 2-butenoate	+		+		+											
Ethyl-3-methyl, 2-butenoate																
Ethylpentanoate	+		+		+						+					
Ethylhexanoate	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
Ethyl-1-methylhexanoate	+															
Ethyl-5-methylhexanoate	+															
Ethyl-2-hexanoate	+		+		+											
Ethyleptanoate	+		+													
Ethyloctenoate	+	+	+	+	+				+		+	+	+	+		+
Ethyl-2-octenoate	+		+													
Ethylnonanoate	+		+													
Ethyldecanoate	+	+	+	+		+			+		+			+		
2-methylpropylbutanoate	+		+													
2-methylpropylesanoate	+		+													
Isoamilacetato	+		+		+											
Isopenthylhexanoate	+		+													
Methyloctanoate	+															
Acids																
Acid 3-methylbutanoic						+				+					+	
Acid 2-methylbutanoic						+				+					+	

+: molecules detected in the headspace of the different samples analysed.

and alcohols showed the highest quantity in sterile and non-sterile meat during air storage (Table 3). The compounds detected in the majority of the inoculated samples stored at 4 °C were acetoin, 2-heptanone, 1-octen-3-ol and 3-methyl-1-butanol. Acetoin was the most commonly found molecule in the headspace of all meat samples showing the highest quantity among the compounds detected. Acetoin concentration increased during storage in all sterile meat inoculated with the seven strains of *B. thermosphacta* compared to the uninoculated control ($P < 0.05$).

High levels of 2-heptanone and 3-octanone were detected in non-sterile meat samples inoculated with strain Dc71. The highest amounts of 1-octen-3-ol were detected in sterile meat inoculated with strains M175 and GC202 and in non-sterile meat inoculated with Dc201 (Table 3).

4. Discussion

The objective of this study was to investigate the spoilage-related activities of different strains of *B. thermosphacta in vitro* and in meat in order to test the individual ability of each strain to produce VOCs during the storage of meat in aerobic conditions.

Strains of *B. thermosphacta* were isolated from all the fresh and spoiled meat samples analysed in this study, identified by Real-Time-PCR and biotyped by RAPD-PCR. None of the strains analysed was able to hydrolyze sarcoplasmic proteins *in vitro* and *in situ* and none showed lipolytic activity either at 4 °C or 20 °C. Reports on the proteolytic and lipolytic activities of *B. thermosphacta* are scarce and ambiguous. Papon & Talon (1988) reported that the optimum temperature for the synthesis of lipases by *B. thermosphacta* is 20 °C. Labadie (1999) reported that *B. thermosphacta* synthesized lipase which preferred glycerol esters and short fatty acids as substrates, while according to Braun & Sutherland (2003) this bacterium is not lipolytic below 20 °C. Similar results were also found by Nowak, Rygala, Oltuszak-Walczak, & Walczak (2012) who described most strains of *B. thermosphacta* with lipolytic activity at 25 °C and only some at 4 °C. The same authors described the inability of the isolates to digest casein or gelatin at either temperatures. Labadie (1999) reported that *B. thermosphacta* did not degrade proteins while Braun & Sutherland (2003) observed that this species synthesized proteolytic enzymes in the stationary phase of growth and exoproteases were not produced below 6 °C. The variable results reported in the literature and in the present study may reflect an intra-species diversity of these activities or the influence of the experimental conditions used.

All the strains of *B. thermosphacta* tested in our study were able to produce histamine and none produced other biogenic amines such as tyramine, cadaverine and putrescine. This result is in agreement with the study by Nowak & Czyzowska (2011) that described 22 strains of *B. thermosphacta* isolated from meat and meat products able to produce histamine and tyramine but neither putrescine or cadaverine. Emborg, Laursen, & Dalgaard (2005) observed no histamine synthesis by five

Table 3
Mean levels of VOCs detected in the headspace of sterile and non-sterile meat samples inoculated with 7 strains of *B. thermosphacta* after 7 days of storage at 4 °C in aerobic conditions.

	VOCs (μg) in inoculated sterile meat[a]						
	DC71	D01	CC75	GC74	M175	DC201	GC202
Ketones							
Acetoin	12315.70 ± 629.92Aa	165.21 ± 2.16Ba	3827.13 ± 13.82C	362.47 ± 12.49D	588.84 ± 7.48Ea	7446.27 ± 33.22Fa	1215.08 ± 153.28Ga
2-heptanone	2.59 ± 0.03Aa	2.74 ± 0.32Aa	0.00	0.00	11.22 ± 0.38B	0.00	0.00
3-octanone	0.00	0.00	0.00	0.38 ± 0.07Aa	178.78 ± 0.51B	0.00	0.00
Aldehydes							
Heptanal	0.00	0.00	0.00	0.00	0.00	0.00	0.17 ± 0.15
Nonanal	0.00	0.00	0.00	0.00	0.18 ± 0.04Aa	0.00	0.01 ± 0.002B
Alcohols							
3-methyl-1-butanol	11.79 ± 3.31Aa	1.17 ± 0.08Ba	0.00	0.00	15.82 ± 0.30A	0.00	0.31 ± 0.040Ca
1-hexanol	40.00 ± 0.96A	0.00	0.00	0.47 ± 0.02B	6.06 ± 0.2C	1.01 ± 0.20D	3.21 ± 2.6Ea
Heptanol	0.00	0.00	0.00	0.00	0.00	0.00	0.05 ± 0.11
1-octen-3-ol	1.93 ± 0.33Aa	0.74 ± 0.21Ba	0.00	0.59 ± 0.11Ba	12.09 ± 0.71C	0.00	6.18 ± 3.1 Da
1-octanol	0.00	0.00	0.00	0.03 ± 0.02Aa	0.10 ± 0.01B	0.12 ± 0.002Ba	0.17 ± 0.014Ca
2-butoxyethanol	9.86 ± 0.56A	0.00	0.00	0.00	0.00	0.00	1.74 ± 0.15B
2-ethyl-1-hexanol	0.00	0.00	0.65 ± 0.05Aa	0.03 ± 0.001Ba	0.12 ± 0.001C	0.00	0.16 ± 0.02Ca
2-ethyl-1-decanol	0.71 ± 0.12a	0.00	0.00	0.01 ± 0.002a	0.16 ± 0.004a	0.12 ± 0.004a	0.00
	VOCs (μg) in inoculated non-sterile meat						
	DC71	D01	CC75	GC74	M175	DC201	GC202
Ketones							
Acetoin	23192.31 ± 612.15Ab	725.63 ± 99.85Bb	0.00	0.00	725.56 ± 7.86Cb	4092.45 ± 12.76Db	547.63 ± 10.34Cb
2-heptanone	346.65 ± 26.33Aa	4.78 ± 0.32Ba	0.00	3.36 ± 0.27B	0.00	0.00	0.92 ± 0.11B
3-octanone	162.18 ± 6.24A	0.00	0.00	0.63 ± 0.24Ba	0.00	0.00	8.45 ± 0.68B
Aldehydes							
Heptanal	29.28 ± 1.66A	0.00	0.00	0.00	0.00	0.81 ± 0.06B	0.00
Nonanal	0.00	0.00	0.00	0.00	0.04 ± 0.001a	0.00	0.00
Alcohols							
3-methyl-1-butanol	263.20 ± 25.41Ab	2.04 ± 0.09Ba	0.00	0.00	0.00	1.32 ± 0.45B	1.80 ± 0.19Ba
1-hexanol	0.00	0.00	0.00	0.00	0.00	0.00	0.61 ± 0.012b
Heptanol	0.00	0.05	0.00	0.00	0.00	0.00	0.00
1-octen-3-ol	0.02 ± 0.04Ab	0.53 ± 0.09Ba	0.00	0.32 ± 0.03Bb	0.00	3.70 ± 0.25Cb	1.89 ± 0.01Bb
1-octanol	0.00	0.02 ± 0.001A	0.00	0.02 ± 0.001Aa	0.00	0.00	0.06 ± 0.021Ab
2-butoxyethanol	19.18 ± 2.04A	0.00	0.00	0.00	0.00	0.92 ± 0.07B	0.00
2-ethyl-1-hexanol	4.61 ± 0.35A	0.00	3.17 ± 0.02Ab	0.17 ± 0.01Bb	0.00	0.00	0.01 ± 0.01Ba
2-ethyl-1-decanol	2.09 ± 0.12Ab	0.00	0.00	0.01 ± 0.002Ba	0.03 ± 0.001Bb	0.18 ± 0.03Ca	0.06 ± 0.01BC

[a] Quantities were determined in the headspace of meat samples analysed as described in Materials and methods. The results are expressed as mean values from 3 replicates of volatile metabolite ± standard deviation. Each reported value is blanked with the corresponding control (uninoculated meat). A–G: for each compound within a row, different letters indicate significant differences ($P \leq 0.05$); a–b: for each compound within a column, different letters indicate significant differences ($P \leq 0.05$).

B. thermosphacta isolates from tuna while Paleologos, Savvaidis, & Kontominas (2004) found *B. thermosphacta* strains able to produce putrescine, tyramine, cadaverine and tryptamine.

The involvement of strains of *B. thermosphacta* in the development of spoilage-related molecules during meat storage was evaluated. Acetoin was the major compound isolated from sterile and non-sterile meat samples both inoculated and not inoculated. High concentration of acetoin was found in all inoculated meat samples while the concentrations of other VOCs such as 1-octen-3-ol, 2-heptanone and 3-methyl-1-butanol appeared as strain-dependent. Some of the VOCs detected in the headspace of meat samples were odour-active molecules possibly responsible for off-flavour release during meat storage at chill temperature. On the basis of the occurrence, acetoin could be regarded as the main spoilage molecule produced by *B. thermosphacta*. Acetoin is considered an important component in terms of flavour as related to a creamy dairy odour (Casaburi et al., 2014; Smit, Smit, & Engels, 2005; Soncin, Chiesa, Cantoni, & Biondi, 2007). It is described as an important contributor to the spoilage of different meat products (Casaburi et al., 2014; Dainty & Hibbard, 1983; Pin et al., 2002) and recently has been used as chemical marker for the sensory shelf-life of saveloy (Holm, Schäfer, Koch, & Petersen, 2013, Holm, Schäfer, Skov, Koch, & Petersen, 2012). The results obtained in the present study highlight the ability of *B. thermosphacta* strains to use mainly glucose as substrate in spoilage producing acetoin as the major end product of the aerobic metabolism of carbohydrate. This is in agreement with Dainty & Hibbard (1983) who described the capability of *B. thermophacta* to grow in meat producing only acetoin and acetic acid from glucose and with other studies that indicated glucose, ribose, glycerol, glycerol-3 phosphate and inosine as substrates used by *B. thermosphacta* growing aerobically in meat (Alexandrakis, Downey, & Scannel, 2012; Holm et al., 2012). The growth of *B. thermosphacta* in meat was associated with the production of acetoin and isobutyric, isovaleric and 2 methylbutyric acids from valine, leucine and isoleucine; all these compounds with sweet, sickly and malty odour (Dainty, Edwards, & Hibbard, 1985). These three acids were not found in the present work probably because the strains tested showed no proteolytic activity. Another important odour-active volatile detected in the headspace of most of the meat samples analysed was 1-octen-3-ol, a common product from lipid oxidation, that was also found in shrimp inoculated with strains of *B. thermosphacta* and *Carnobacterium maltaromaticum* (Laursen, Leisner, & Dalgaard, 2006) reported as key odorant in most cheeses and main odour-active compound of soft cheese (Curioni & Bosset, 2002). Although this compound is produced in much lower quantities than acetoin, it is considered an odour-active molecule possibly responsible for off-flavour release during meat storage at chill temperature because the odour threshold value (about 0.0075 ppm) is low (Calkins & Hodgen, 2007).

The role of *B. thermosphacta* as potential spoiler of meat did not appear to be influenced by indigenous microbiota of the meat. Inoculation with *B. thermosphacta* of meat samples determined a significant increase of acetoin during aerobic storage. The strains of *B. thermosphacta* analysed in this study were unable to degrade meat proteins and lipids, whose degradation is usually associated with organoleptic changes. On the basis of our results, the role of *B. thermosphacta* as aerobic spoiler of meat can be due to the consumption of glucose with production of acetoin, described as important contributor to the spoilage of different meat products. Further studies are necessary on the effect of this compound on the sensory quality of fresh meat stored at chill temperature in order to further support the role of acetoin producers as spoilage bacteria in meat.

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