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Genotypic characterization of *Brochothrix thermosphacta* isolated during storage of minced pork under aerobic or modified atmosphere packaging conditions

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Abstract

A total of 306 colonies were isolated from the selective medium for *Brochothrix* spp., during the spoilage process of minced pork stored at 0, 5, 10 and 15 °C and packed aerobically and under MAP. *Brochothrix* biodiversity was assessed by Pulsed Field Gel Electrophoresis (PFGE), and representative strains were further analysed by Rep-PCR using primer $(GTG)_5$ and Sau-PCR with primers $SAG_1$and SAG_2. Although, different results were obtained from the different methods applied, a significant diversity among isolates recovered from aerobic conditions was observed. On the contrary, isolates from MAP showed a lower degree of heterogeneity. The storage conditions affected the *Brochothrix* diversity, being the strains isolated in the initial stage different from the ones present at the final stage of storage at chill temperatures. A representative number of isolates, based on the results of the clustering by molecular methods, was subjected to 16S rRNA gene sequencing, revealing that all belonged to *Brochothrix thermosphacta*.

Keywords: pork, *Brochothrix thermosphacta*, PFGE, Rep-PCR, Sau-PCR

1. Introduction

It is generally accepted that bacteria are absent, undetectable or extremely low in muscle tissues of healthy live food animals (Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). As the inherent protective barriers (skins, hides) and the natural antimicrobial defence mechanisms (lysozyme, antimicrobial peptides) of the live animal are destroyed at slaughter, the resulting meat becomes exposed to increasing levels of contamination and depending on various parameters, undergoes rapid microbial decay. Sources of these microorganisms include gastrointestinal tract and the external surfaces of the animal including the environment with which the animal had contact some time before or during slaughter. Among these bacteria *Enterobacteriaceae, Brochothrix thermosphacta, Pseudomonas* spp., and lactic acid bacteria (LAB) have been found to be major contributors to meat spoilage, depending on product type and conditions surrounding the product (Nychas et al., 2008). For example, it is well established that, *Pseudomonas* spp. is the dominant microbiota of meat stored under aerobic conditions, while *B. thermosphacta* becomes the dominant bacterium along with LAB throughout storage of meat stored under modified atmosphere (Nychas et al., 2008; Pennacchia, Ercolini & Villani, 2011; Doulgeraki, Paramithiotis, Kagkli & Nychas, 2010; Russo, Ercolini, Mauriello & Villani, 2006; Xu, Anyogu, Ouoba & Sutherland, 2010). In particular *B. thermosphacta,* with no proteolytic activity, has been found to produce a number of compounds e.g. acetoin, acetic, isobutyric, 2-methylbutyric, isovaleric acids, lactic acid, carbon dioxide and ethanol under aerobic or limited oxygen concentration, that contribute to meat with a distinct spoilage type, as it was reposted in studies where raw meat stored under oxygen limitations (Dainty 1996; Dainty & Mackey, 1992; Nychas et al., 2008; Russo, et al., 2006). Moreover, discoloration and gas production can occur. In comparison with pseudomonads, *Enterobacteriaceae* and lactic acid bacteria studies the genotype

differences between strains of *B. thermosphacta* have been only limited studied (Xu, et al., 2010).

Nowadays, the application of molecular techniques has become a routine step for identification properties, both in the identification of pathogenic microorganisms (Wang, Li & Mustapha, 2007) and spoilage bacteria. However, to our knowledge only a few studies in the field of meat/food have been published focusing on the characterization of *B. thermosphacta*. Pennacchia, Villani & Ercolini, (2009) employed quantitative PCR for the detection, identification and quantification of *B. thermosphacta* from meat in an attempt to shorten the time requested by microbiological analysis. Xu et al. (2010) studied strains of *Brochothrix* spp. which had been isolated from fish, meat and poultry using PCR amplification of 16S-23S rDNA intergenic transcribed spacer (ITS-PCR), repetitive sequence-based PCR (rep-PCR) and 16S rDNA sequencing and their findings showed minor genotypic differences between those strains. In all of the cases *Brochothrix* spp. was identified as *B. thermosphacta*. Similar results were reported by Pennacchia et al. (2011) in studies on chilled beef packaged under air or vacuum pack. However, in a recent research (Nowak & Piotrowska, 2012), it was reported that different strains of *B. thermosphacta* produce different hydrolases, which participate in the spoilage process of meat and meat products. In more detail, some strains are capable of protein degradation or produce proteases with different substrate specificity. Thus, the exploration of succession of strains that prevail and contibuting in spoilage progress of meat would expand knowledge of meat microbial ecology and consequently understanding the meat spoilage process.

The aim of the present study was not only to determine the genotypic diversity of *Brochothrix* isolates recovered from minced pork stored under different packaging and temperature conditions, but also to monitor the succession of these strains during different storage conditions. To achieve these goals three different methods, namely Repetitive

Extragenic Palindrome (Rep)-PCR, Sau-PCR and Pulsed field gel electrophoresis (PFGE) were used for differentiation of the isolates.

2. Materials and Methods

2.1 Sample separation and storage conditions

Fresh minced pork (*Biceps femoris*, pH 5.6-5.8) was purchased immediately after grinding from the central meat market in Athens and transported under refrigeration to the laboratory within 30 min. Minced pork was prepared according to Papadopoulou, Panagou, Tassou, & Nychas (2011). Briefly, the meat was divided in portions of 50 g and placed in Styrofoam trays which subsequently packed under aerobic modified atmosphere packaging (MAP, 60% CO_2/20%O_2/20% N_2). They were stored at 0, 5, 10 and 15 °C until spoilage was very pronounced. For aerobic storage the trays were wrapped manually with air-permeable polyethylene plastic film, while in the case of MAP, samples were packed and sealed into plastic pouches with gas permeability at 20 °C and 50% relative humidity of ca. 25, 90 and 6 cm^3m^{-1} per day/10^5 Pa for CO_2 and N_2 respectively, using a HankoVac 1900 Machine (Howden Food Equipment B.V., The Netherlands).

2.2 Isolation and growth of *Brochothrix* spp.

Colonies were isolated during storage of minced pork meat according to Doulgeraki et al., (2010). In brief, minced pork was sampled at appropriate time intervals, depending on storage temperature and packaging conditions. Under aerobic conditions, the incubation lasted 340, 243, 97 and 92 hours, while under MAP lasted 579.5, 411, 199 and 101 hours at 0, 5, 10 and 15 °C, respectively. From different time points (fresh meat, middle and final stage of storage) approximately 40 colonies were selected randomly from the highest dilution of Streptomycin

Thallous Acetate-Actidione Agar (STAA, Biolife Italiana S.r.l., Milano, Italy). Pure cultures included in this study, were stored at −80 °C in Brain Heart Infusion Broth (BHI, Merck, Darmstadt, Germany) supplemented with 20% (v/v) glycerol (Merck, Darmstadt, Germany) . Before further analysis, each isolate was grown twice in BHI broth at 25 °C for 24 h. Purity of the culture was always checked on BHI agar plates before use.

2.3 PFGE

PFGE was performed according to Doulgeraki et al. (2010). The restriction enzyme *Apa*I (10 U) (New England Biolabs, Ipswich, MA, USA) was used according to the manufacturer's recommendations for 16h. Restriction fragments were separated in 1% PFGE grade agarose gel (Bio-Rad, Hercules, CA, USA) in 0.5 mMTris-Borate buffer on CHEF-DRII (Bio- Rad) equipment with the following running parameters: 6 V cm^{-1}, 4 sec initial switching time, 40 sec final switching time and 18 h of a total run at 14°C. Gels were then stained with ethidium bromide (0.5 mg ml^{-1}) in water for 1 h and destained for 2 h before being photographed using a GelDoc system (Bio-Rad). Normalization and further analysis were performed using the Pearson coefficient and UPGMA cluster analysis with Bionumerics software, version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium).

2.4 DNA extraction from pure cultures

The cells from 1mL of a 24 h culture in BHI broth (Merck) were collected after centrifugation at 14,000 x g for 10 min. DNA was extracted according to Cocolin, Stella, Nappi, Bozzetta, Cantoni, & Comi (2005).

2.5 Rep-PCR analysis

One hundred nanograms of the DNA extracted from isolates were subjected to Rep-PCR analysis using primer $(GTG)_5$ (5'-GTGGTGGTGGTGGTG-3') according to Iacumin,Comi, Cantoni, & Cocolin, (2006). Reactions were carried out in a final volume of 25 µl containing: 10 mM PCR Gold Buffer 10X, 25 mM $MgCl_2$, 10 mM mix dNTPs, 10 µM primer $(GTG)_5$ and 5 U μL^{-1} Taq-polymerase (Applied Biosystem, Carlsbad, USA). PCR reactions consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 90°C, 30 sec, primer annealing at 40°C, 1 min and primer extension at 65 °C, 8 min, and concluded by a final extension step at 65°C for 16 min. Amplicons were separated in a 2% (w/v) agarose gel in TBE 1X at 120 V for 2 h. After the run, gels were stained with ethidium bromide 0.5 µl mL^{-1} for 30 min. Normalization and further analysis were performed using the Pearson coefficient and UPGMA cluster analysis with Bionumerics software, version 6.1.

2.6 Sau- PCR

One hundred nanograms of the DNA extracted from isolates were subjected to digestion using the *Sau*3A (10 U μl^{-1}) restriction endonuclease (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's recommendation, in a total volume of 20 µl. After an overnight period at 37 °C, 1 µl was transferred into a 25 µl reaction final volume containing PCR-Gold Buffer 10X, 25 mM $MgCl_2$, 10 mM mix dNTPs, 2µM each primer SAG1 (5'-CCGCCGCGATCAG- 3'), SAG2 (5'-CCGCCGCGATCTC-3'), $5U\mu L^{-1}$ Taq-polymerase (Corich,Mattiazzi, Soldati, Carraro & Giacomini, 2005). PCR reactions were carried out using the following amplifications conditions: 25 °C for 5 min, ramp to 60 °C at 0.1 °C/s, 60 °C for 30 sec, 95 °C for 1 min, 50 °C for 15 sec, ramp to 25 °C at 0.1 °C/sec, ramp to 50 °C at 0.1 °C/sec, 50°C for 30 sec, 35 cycles of 95 °C for 15 sec, 46 °C for 1 min, 65 °C for 2 min, and the final extension at 65 °C for 5 min. Amplicons were separated in a 2% (w/v) agarose gel in TBE 1X at 120 V for 2 h. After the run, gels were stained with ethidium

bromide 0.5 mg mL^{-1} for 30 min. Normalization and further analysis were performed using the Pearson coefficient and UPGMA cluster analysis with Bionumerics software, version 6.1.

3. Results and Discussion

Spoilage progress of meat and meat products have been reported extensively (see Dainty & Mackey 1992; Dainty, 1996, Nychas et al. 2008). In these reviews as well as in other studies, the diversity of the microbial population in meat and meat products stored under various conditions has been described in detail (Doulgeraki et al, 2010; Doulgeraki, Paramithiotis, & Nychas, 2011; Cocolin, Rantsiou, Iacumin, Urso, Cantoni, & Comi, 2004; Ercolini et al., 2006, Ercolini, Russo, Nasi, Ferranti, & Villani, 2009, Ercolini, Ferrocino, La Storia, Mauriello, Gigli, Masi, Villani, 2010; Stanbridge & Davies, 1998; Vasilopoulos, Ravyis, De Maere, De Mey, Paelinck, De Vuyst, & Leroy, 2008, Vasilopoulos, De Maere, De Mey, Paelinck, De Vuyst, & Leroy, 2010). However, the systematic study of the succession of members of the microbial association as well as the changes occurring at strain level, indicated as Ephemeral Spoilage Organisms (ESO), only recently was taken into account (Doulgeraki et al., 2010; Nychas et al, 2008). It is widely accepted that *B. thermosphacta* is recognised as significant portion of the spoilage microbiota of meat (Enfors, Molin, & Ternstroem, 1979; Lambropoulou, Drosinos, & Nychas, 1996; Newton & Gill, 1978; Nychas &Skandamis, 2005; Pennacchia et al., 2011; Russo, et al, 2006; Xu et al, 2010), however limited information are provided in the literature. Similarly, *Brochothrix* was found to be the dominant population, along with LAB in samples stored under modified atmospheres, while on samples stored under aerobic conditions *Pseudomonas* spp. were the dominant biota followed by *Brochothrix* (detailed description of microbial association is presented elsewere, Papadopoulou et al., 2011). Table 1 summarizes the STAA counts of fresh minced pork and at two different stages of storage (middle and final) for each condition adopted. In the case of

chill temperatures (0 and 5 °C), bacterial counts were lower at final stages of storage when samples were stored under MAP, while at abuse temperatures (10 and 15 °C), counts were quite similar for both aerobic conditions and MAP.

In the present study the diversity as well as the succession of *B. thermosphacta* at strain level from minced pork stored under different packaging (air and MAP) and temperature (0, 5, 10 and 15 °C) conditions is shown. It is also clear in this study that the methodology used to either enumerate or study the microbial association deeply influence the outcome. Thus, 306 *Brochothrix* isolates were recovered from fresh minced pork and at two time intervals (middle, final stage) of the storage period, from all conditions adopted (155 isolates from aerobic conditions and 151 from MAP), differentiated according to their genotypic profile (PFGE, Rep-PCR and Sau-PCR) and identified by sequencing of the V1–V3 variable region of the 16S rRNA gene. The ability of these methods to provide characterization at sub-species or strain level was based on different genomic elements. PFGE is able to produce patterns related to the DNA fragments resulting from the digestion of whole genomic DNAs with rare-cutting restriction endonucleases. Rep-PCR produces profiles related to the presence of repeated sequences within the genomes, while Sau-PCR fingerprints are created based on the presence on the bacterial genome of restriction sites for enzyme *Sau3*A.

Different results were obtained from the different methods applied, which were mostly attributed to the degree of discrimination of the different technique. Indeed, the macrorestriction analysis of DNA of *B. thermosphacta* isolates by PFGE resulted in 4 clusters, which were able to distinguish the isolates detected at 0°C from the rest, as well as the isolates obtained from the different packaging at 0°C. The efficacy of the latter method to differentiate the isolates based on the specific conditions adopted has been previously reported in the case of minced beef (Doulgeraki et al. 2010, 2011). Similarly, the results

obtained applying the Rep-PCR method was interesting, as after cluster analysis of the different profiles, two clusters consisted of isolates detected under aerobic conditions. Rep-PCR has been previously found able to form different clusters for isolates recovered from different processing plants (Iacumin et al., 2006) and different food samples (Xu et al. 2010). On the other hand, cluster analysis obtained from Sau-PCR, with a coefficient of similarity 70% did not give similar results as the two aforementioned molecular techniques. This could be possibly explained by the fact that a total of 33 different fingerprints were resulted after image analysis.

The dendrogram obtained after cumulative image analysis of PFGE, Sau-PCR and Rep-PCR patterns using a coefficient of similarity of 86%, resulted into 8 different groups (data not shown). In Table 2, the prevalence of the different groups related to the different storage conditions is summarized. More specifically, group I consisted of isolates recovered from fresh meat and middle stage of storage at 0°C from aerobic conditions. Group II consisted of 3 isolates recovered from the final stage of storage at 10°C and 1 isolate from fresh meat under aerobic conditions. Group III consisted of a single isolate recovered from middle stage of storage at 0°C in aerobiosis. Group IV, encompassed strains of middle and final stage of storage at 0°C in MAP, and 3 isolates which were recovered from the portion of fresh meat that packaged under modified atmosphere. Group V consisted of 3 strains isolated at middle stage of storage at 0°C and 1 strain at 10°C from aerobic conditions. The next group (VI) consisted of a total of 63 isolates; 29 and 34 of them recovered from aerobic conditions and MAP, respectively. More specifically under aerobic conditions group VI was detected on fresh meat, middle of storage at 5 and 10°C and final stage of storage at 0, 5 and 10°C, while in the case of MAP it included isolates from fresh meat, middle stage of storage at 0, 5 and 10°C, and final stage of storage at 10°C. On the other hand, in group VII, isolates from fresh meat (air and MAP), and isolates from 5°C and 15°C under aerobic conditions and

MAP, respectively, were clustered. Finally, group VIII was detected in all conditions adopted but one; at 5°C under MAP. Additionally, the aforementioned group was the most common one for fresh meat, 5°C under aerobic conditions, 10°C under MAP (final stage of storage) and 15°C under aerobic storage (final stage) and MAP. In the latter case (15°C, MAP), group VIII was the only Group detected. A representative number of isolates from the above groups was subjected to 16S rRNA gene sequencing, revealing that all groups were assigned to *Brochothrix thermosphacta* (data not shown).

A significant diversity among isolates recovered from aerobic conditions was observed while for isolates from MAP, the diversity was lower. These results were not in line with previous studies were MAP found to be able to increase both species and strain diversity (Doulgeraki et al. 2010, 2011). However, the storage conditions affected the strain diversity, although similar counts were detected. Additionally, the populations recovered from the initial stage were markedly different from the ones at the end of the storage at chill temperatures. Similar observations have been previously reported for beef (Doulgeraki et al. 2010, 2011; Ercolini et al., 2006). Indeed, cluster VIII, which was the most abundant, was formed from isolates recovered from fresh meat and abuse temperatures (10 and 15°C). It has to be noted that all isolates detected at 15°C under MAP were clustered together in the latter group. Furthermore, in the case of chill temperatures, the packaging conditions affected the strain diversity as the isolates were clustered in different groups. Indeed, isolates recovered from 0°C were clustered in cluster VI and IV under aerobic and MAP conditions, respectively.

The present study does provide an insight into the *B. thermosphacta* strain diversity in relation to temperature and packaging as it was shown that *B. thermosphacta* strains differ between these conditions. These results are of great importance, since it can be considered that they widening the knowledge of meat microbial ecology and specifically do contribute to

a better understanding of spoilage process. The strain diversity indicated in this study, should be further studied e.g. the biochemical characteristics (sacharolytic, lipolytic activity, etc) of the prevailing species or strains under different conditions. Similar observations and conclusion have been reported with other species and/ or strains of e.g. LAB, *Enterobacteriaceae* and pseudomonads (Doulgeraki et al. 2010, 2011; Ercolini et al. 2006, 2009, 2010; Pennacchia et al. 2011).

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Tables

Table 1. STAA populations in minced pork meat stored under aerobic and MAP conditions

Temperature °C	Storage period	STAAcounts (log CFU g^{-1})[a]	
		Air	MAP
0	Initial	4.03±0.70	4.15±0.48
	Middle	6.77±0.01	5.90±0.32
	Final	8.12±0.11	6.10±0.04
5	Middle	7.64±0.15	6.64±0.06
	Final	8.07±0.31	7.00±0.05
10	Middle	6.64±0.13	6.51±0.72
	Final	7.66±0.11	7.17±0.42
15	Middle	7.45±0.64	6.79±0.05
	Final	7.96±0.16	7.46±0.17

[a]*Brochothrix* counts are presented as mean ± standard deviation.

Table 2. Distribution of *Brochothrix thermosphacta* isolates during spoilage of minced pork meat at 0, 5, 10 and 15°C under aerobic and modified atmospheres

Packaging	T (°C)	Total isolates	Group[a]							
			I	II	III	IV	V	VI	VII	VIII
AIR	Fresh meat	39	9	1	-	-	-	1	8	20
	0	29	7[b]/0[c]	-	0/1	-	3/0	0/10	-	4/4
	5	30	-	-	-	-	-	1/1	-	14/14
	10	27	-	0/3	-	-	1/0	7/9	-	6/1
	15	30	-	-	-	-	-	-	15/4	0/11
MAP	Fresh meat	38	-	-	-	3	-	12	4	19
	0	30	-	-	-	8/15	-	1/0	-	6/0
	5	25	-	-	-	-	-	2/0	8/15	-
	10	28	-	-	-	-	-	15/4	-	0/9
	15	30	-	-	-	-	-	-	-	15/15
Total isolates		306	16	4	1	26	4	63	54	138

[a] according to PFGE, Sau-PCR and Rep-PCR patterns
[b] middle time of storage
[c] final time of storage