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Development of a Real-Time PCR assay for the specific detection of *Brochothrix thermosphacta* in fresh and spoiled raw meat

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ABSTRACT

Brochothrix thermosphacta is a psychrotrophic species commonly involved in the spoilage of meat and often recognized as the dominant organism causing off-flavours. The knowledge of the genera/species affecting meat spoilage is necessary to define a successful method for food preservation. The aim of this study was to develop a Real-Time (RTi-) PCR method for the species-specific detection of *B. thermosphacta* and to evaluate a RTi-PCR approach for its enumeration in fresh and spoiled beef, avoiding the culturing steps. The specificity of the primers designed on the basis of the 16S rRNA gene sequences of *B. thermosphacta* was tested using the DNA extracted from strains belonging to bacterial species usually associated with meat. The RTi-PCR assay allowed a species-specific detection of *B. thermosphacta* and no amplification signals were retrieved using DNA from the other species under the conditions used.

Three different standard curves were constructed by using broth culture, a meat extract and meat samples containing different concentrations of *B. thermosphacta*. The standard using artificially contaminated meat samples was chosen because of its closeness to an authentic contamination case. The standard curve was linear in the range from 2.2×10^2 to 2.3×10^7 CFU/g; the reaction efficiency was 1.11. The RTi-PCR assay was then applied to enumerate *B. thermosphacta* in 20 fresh and spoiled beef samples and the results were compared to those obtained by plating onto selective medium for *B. thermosphacta*. A comparison between the two methods reported a general underestimation (from 0.5 to 2 Log CFU/g) of the microbial loads by RTi-PCR. Except for a few cases, the statistical analysis showed significant differences between viable counts and RTi-PCR data.

The identification of *B. thermosphacta* by the RTi-PCR method developed in this study is certainly simple and fast and can be useful for its reliable detection in meat samples. However, considering the level of underestimation reached in most of the samples analyzed, the RTi-PCR method can be recommended only to approximately predict the contamination level of *B. thermosphacta* in meat.

1. Introduction

Meat represents a complex ecosystem with particular physical and chemical characteristics which can allow the colonization and the development of a great variety and number of organisms (Ercolini et al., 2006; Nychas et al., 2008). Several studies on the microbial spoilage ecology have recognized *Brochothrix* (*B.*) *thermosphacta*, *Pseudomonas* spp., *Carnobacterium* spp., *Enterobacteriaceae*, *Lactobacillus* spp., *Leuconostoc* spp. and *Shewanella putrefaciens* as the predominant members of spoilage microflora in refrigerated meat and meat products, such as beef and pork (Borch et al., 1996; Barakat et al., 2000; Ercolini et al., 2006; Nychas et al., 2008; Ercolini et al., 2009).

Spoilage is defined as any change in a food product that makes it unacceptable to the consumer from a sensory point of view (Gram et al., 2002).

The psychrotrophic species *B. thermosphacta* represents a significant component of the spoilage microflora of meat stored aerobically and occasionally it is recognized as the dominant organism. For this species, meat is an ecological niche where it can grow under both aerobic and anaerobic conditions and produce undesirable odours (Dainty et al., 1985; Labadie, 1999; Pin et al., 2002). *B. thermosphacta* can be source of off-flavour producing cheesy odours associated with acetoin/diacetyl and 3-methylbutanol production (Borch and Molin, 1989; Dainty and Mackey, 1992).

Numerous preservation methods have been applied, individually or in combination, to extend the shelf-life of meat during storage without the use of chemical preservatives (vacuum or modified atmosphere packaging). Food preservation and the product type affect the growth rate and the composition of spoilage microflora during refrigerated storage (Dykes et al., 1996; Franz and von Holy, 1996;

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Samelis et al., 2000; Ercolini et al., 2006); therefore, the knowledge of the genera/species affecting meat spoilage is necessary to establish methods and storage conditions effective in food preservation (Macian et al., 2004; Ercolini et al., 2006; Ercolini et al., 2009).

The common methods used to detect, enumerate and/or identify microorganisms in foods are generally based on conventional microbial techniques; they are often time consuming and may bias the real composition of the spoilage bacterial population (Macian et al., 2004; Ercolini, 2004; Ercolini et al., 2006; Rodríguez-Lázaro et al., 2007).

PCR is a molecular diagnostic tool able to significantly reduce the time necessary for detection and screening of foods for spoilage and/or pathogenic bacteria (McKillip and Drake, 2004). In this context, RTi-PCR represents an innovative technique able to allow an accurate and unambiguous identification of microorganisms and a quantification of their nucleic acids, avoiding post-PCR steps with cross-contamination risks (Klein, 2002; Rodriguez-Lazaro et al., 2004). RTi-PCR could offer significant advantages for the enumeration of bacteria directly from food samples and it is widely used in food microbiology (Hanna et al., 2005; Klein, 2002; McKillip and Drake, 2004; Rodríguez-Lázaro et al., 2007).

Recently, RTi-PCR has been employed in food to detect and sometimes to quantify several pathogenic bacteria such as *Salmonella*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Shigella*, *Campylobacter jejuni*, *Clostridium tyrobutyricum*, and *Yersinia enterocolitica* (D'Urso et al., 2009; Rantsiou et al., 2008; López-Enríquez et al., 2007; Ronner and Lindmark, 2007; Wang et al., 2007; McKillip and Drake, 2004; Rodriguez-Lazaro et al., 2004; Wolffs et al., 2004). In order to detect a small number of target cells, some methods require enrichment procedures prior to RTi-PCR (Fujikawa and Shimojima, 2008). In such cases, the RTi-PCR approach is effective but still only qualitative; moreover, it does not take into account the complex microflora often contaminating the raw foods which could interfere with the detection of the target bacterium (Fujikawa and Shimojima, 2008).

In the present work, we describe a new species-specific RTi-PCR assay for the detection and identification of *B. thermosphacta*. Moreover, we evaluate a culture-independent RTi-PCR approach for a quantitative detection of *B. thermosphacta* in order to avoid the time consuming steps of microbiological analysis.

2. Materials and methods

2.1. Bacterial strains and DNA extraction

Bacterial strains used in this study are listed in Table 1. DNA from each strain was extracted from a culture grown in 10 ml of Tryptone Soya Broth (TSB, Oxoid, Garbagnate Milanese, Italy) with 0.5% yeast extract (Oxoid) after an aerobic incubation for 24–48 h at its optimal growth temperature (Table 1). After incubation and prior the DNA extraction, a preliminary treatment with Propidium Monoazide (PMA) was carried out in order to avoid detection of dead cells (Pan and Breidt, 2007). Twenty microliters of PMA (2.5 mM in 20% dimethyl sulfoxide) were added to 980 µl of bacterial culture and incubated for 5 min at room temperature in the dark. The sample was exposed for 5 min to a 600-W halogen light source placed 20 cm directly above the open vial on chipped ice, as suggested by Pan and Breidt (2007). After the treatment, the culture was subjected to DNA extraction by using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions and by the Wizard DNA purification kit (Promega, Madison, Wiscon.) as reported by Villani et al. (2007).

Table 1
Microbial strains used in this study.

Species	Strain	Source[a]	Temperature of incubation
Brochothrix thermosphacta	1R2	DSA	25 °C
Brochothrix thermosphacta	3R2	DSA	25 °C
Brochothrix thermosphacta	7R1	DSA	25 °C
Carnobacterium divergens	3P	DSA	25 °C
Carnobacterium maltaromaticus	9P	DSA	25 °C
Escherichia coli O157:H7	35/55	DSA	30 °C
Hafnia alvei	53 M	DSA	25 °C
Lactobacillus curvatus	BVL17	DSA	30 °C
Lactobacillus sakei	DVL17	DSA	30 °C
Listeria innocua	1770	DSA	30 °C
Listeria monocytogenes	CAL	DSA	30 °C
Pseudomonas fragi	DSM3456^T	DSMZ	25 °C
Serratia proteamaculans	20P	DSA	25 °C

[a] DSMZ, Deutsche Sammlung von Mikroorganismen und Zelkulturen, Braunschweig, Germany; DSA, Dipartimento di Scienza degli Alimenti, Università degli Studi di Napoli Federico II, Portici, Italy.

DNA extraction from meat samples was performed as above described by using 1 ml of the first serial decimal dilution in quarter strength Ringer's solution (Oxoid) prepared for the viable counts of the beef samples analyzed in this study.

The DNA extraction method used for all the experiments employed the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre) with PMA pre-treatment, since better results were obtained with this kit compared to the Wizard DNA Purification Kit in terms of efficiency, linearity range and quantification limit.

2.2. Primer design and specificity of the assay for the detection of *B. thermosphacta*

The primers Bcr3r (5′-GTT GTC CGG AAT TAT TGG G-3′) and Bcr3f (5′-CTC CTC TTC TGT CCT CAA G-3′) used in this study were designed on the basis of the comparison of the16S rRNA gene sequences of *B. thermosphacta* and of the species listed in Table 1. Sequence alignment was performed by MacDNasis Pro v3.0.7 (Hitachi Software Engineering Europe S.A., Olivet Cedex, France). The alignment is showed in Fig. 1, where the sequence variability used for the species-specific design of the primers is highlighted. The expected size of the PCR product was of 121 bp. The RTi-PCR assay was tested using the strains listed in Table 1. The amplification reactions for standards and commercial meat samples were performed in a total volume of 25 µl and every sample was processed in triplicate. Each Real-Time PCR reaction tube contained 12.5 µl of iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA94547, catalog 170-8882), 0.2 µM of each primer and 1 µl (50 ng) of extracted sample DNA. The experiments were performed in the Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories, Milan, Italy), using the following program: 3 min at 94 °C, 30 cycles of 15 s at 94 °C and 30 s at 63 °C. Melting curve was determined at the end of the described program by the following steps: 60 °C to 90 °C, read every 0.5 °C, hold 10 s. A response was considered positive if the amplification curve of two from three replicates crossed the fluorescence threshold line, which was positioned by a background-based algorithm calculated by the software.

2.3. Artificial contamination of beef for RTi-PCR standard curves construction

2.3.1. Contamination of a sterile beef extract

The method described by Martin et al. (2006) was used with some modifications. Briefly, 10 g of raw beef were diluted (1:10) in 90 ml of Ringer solution and homogenized for 1 min in a stomacher bag and preliminarily filtered through a cotton wrap. The homogenate obtained was filtered twice with 0.45 µm and 0.22 µm syringe filters. The filter-sterilized extract was artificially inoculated with decreasing amounts of a 36 h culture of *B. thermosphacta* 1R2 to obtain a range of 1×10^1–1×10^7 CFU/ml. In parallel, viable counts of *B. thermosphacta* 1R2 in each contaminated sample were determined by spread plating onto STAA. The contaminated samples were subjected to DNA extraction as described above. The experiment was repeated three times in triplicate.