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# Characterization of clostridial species and sulfite-reducing anaerobes isolated from foie gras with respect to microbial quality and safety

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ABSTRACT

Anaerobic sulfite-reducing bacteria are generally considered as indicators of clostridial contamination in meat products. We reconsidered the relevance of this indicator for crude and pasteurized foie gras. A three-year study was conducted to recover anaerobic sulfite-reducing bacteria from foie gras manufactures. Under anaerobic culture at 37 °C, 98 isolates were obtained from crude or pasteurized foie gras, surfaces and pepper used in the recipe and further identified. Heat-treated products lead to the exclusive isolation of *Clostridium* strains, but other samples demonstrated a high diversity of non-spore forming bacterial species. The diversity in the *Clostridium* group was also high, with 14 different species represented from the 49 clostridial isolates, including *Clostridium perfringens* and psychrotolerant species. All the 12 *C. perfringens* isolates belonged to type A, but none carried the enterotoxin gene. In addition, none of them was able to grow in foie gras at 8 °C over a 130-days period. Other species were also tested for their ability to grow in these conditions: only three isolates, identified as *Clostridium sordellii, Clostridium tertium* and *Clostridium algidicarnis/putrefaciens*, grew at 8 °C, exhibiting 5 to 6 log of population increase in 70 days. Consequently, regarding the risk of product spoilage, we recommend anaerobes enumeration at 37 °C for pasteurized foie gras products, or after a thermal treatment aiming at spore selection, for crude foie gras.



## 1. Introduction

Foie gras is a typical French food product made from the liver of a duck or goose that has been specially fattened, usually with corn. Liver is intended to be cooked and then stored refrigerated for several months. Vegetative cells are inactivated by the process and thus, the main issue is to control spore forming bacterial growth during storage. In fact, bacterial spores could contaminate the product, pass through the pasteurization process, and could be able to develop during refrigerated storage. During liver preparation, contamination by *Clostridium* spp may occur from multiple sources: animal commensal microflora, animal and manufacture environments, dust, human carriage and spices (Bauer, Carpenter, & Jo, 1981; Boerema, Broda, & Bell, 2002; Broda, Boerema, & Brightwell, 2009; Carlin, 2011; Dodds, 1993; Gibson, Macfarlane, & Cummings, 1988; ICMSF, 1998). Pathogen risk is controlled by the respect of pasteurization procedures and refrigeration temperatures. In addition, both minimization of carcasses contamination by soil and animal faeces and Good Manufacturing Practices are recognized as efficient ways to minimize bacterial spore contamination of foods (EFSA, 2005). Periodical analyses are defined in all manufactures to monitor contamination levels. Classically, two microbial indicators are used to assess product quality in relation to spore-forming bacteria: sulfite-reducing anaerobes (SRA) or *Clostridium perfringens*. Both are widely used as indicators of clostridial contamination in meat products. However, the bacterial group of SRA is not supported by any taxonomical consideration, but only by common phenotypic traits. SRA quantitative determination may thus lead to an overestimation of putative pathogens or spoilage spore-formers. On the other hand, *C. perfringens* analysis would probably not be fully representative of spoilage risks. Furthermore, this analysis requires expensive additional confirmation assays.

The present study aimed to a better characterization of SRA detected from foie gras products and manufacture environments. Health and spoilage risks associated with the detection of clostridial SRA were further investigated.

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## 2. Materials and methods

### 2.1. Sample collection

Hygienic surveys performed on pasteurized foie gras by French manufactures in their specific sampling scheme resulted in 93 samples leading to SRA positive analyses. The samples corresponded to 44 crude foies gras, 35 pasteurized foies gras, two sliced pasteurized foies gras further vacuum packed, seven surfaces of equipments used for foies gras preparation, and 5 peppers. Six crude and one pasteurized foies gras originated from goose liver, while all others were from duck.

### 2.2. Enumeration, isolation, culture and sporulation of anaerobic sulfite-reducing bacteria

Anaerobiosis was obtained by an anaerobiosis generator system (anaerogen 3.5L, Oxoid, Dardilly, France) in a locked jar. Anaerobiosis was verified with anaerobic indicators (Biomerieux, Craponne, France).

SRA were classically enumerated according to the international standard ISO 15213:2003 (ISO, 2008). Briefly, black colonies forming unit were enumerated from poured-plates of ready-to-use tryptose-sulfite agar that contains sodium metabisulfite and ferric ammonium citrate (Biokar diagnostics, Beauvais, France), incubated 48 h at 37 °C under strict anaerobiosis. From these samples, bacteria leading to black colonies were isolated by successive culture steps on meat-liver agar after 48 h of anaerobic incubation at 37 °C. Isolate purity was admitted when a single black colony phenotype was observed on an isolation plate and further checked by the quality of 16S rRNA region sequence. All subsequent enumerations were performed on meat-liver agar (Biokar diagnostics, Beauvais, France) incubated 48 h at 37 °C under anaerobiosis.

Cultivation steps for foie gras inoculation experiments were performed in Rosenow broth (Bio-Rad, Marnes la Coquette, France), incubated 48 h at 37 °C under anaerobiosis.

Spores were obtained from tryptose-sulfite agar after a 21-day period of incubation at 37 °C under anaerobiosis. Spores were collected, heat treated at 70 °C for 10 min, and rinsed twice with water before storage at 4 °C.

### 2.3. Identification by sequence analysis

Total bacteria DNA was isolated from fresh colonies using the InstaGene® matrix (Bio-Rad, Marne la Vallée). The 16S rRNA region was amplified with FD1 and RD1 universal primers as previously described (Weisburg, Barns, Pelletier, & Lane, 1991). The PCR product was sequenced by Eurofins MWG Operon (Ebersberg, Germany). Identification was proposed from alignments searches with RDP (Ribosomal Database Project; http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) and NCBI Nucleotide Collection NR (Blast tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequences were deposited to GenBank (Genetic sequence database at the National Center for Biotechnology Information (NCBI); GenBank ID: BA123456). They received the accession numbers JX267045 to JX267140.

Shannon's diversity index ($H$) was calculated according to:

$$H = \sum_{i=1}^{s} -(P_i * \ln P_i)$$

with $Pi$ = the number of isolates from the analyzed population that belong to species $i$ divided by the total number of strains, and $S$ = the number of species encountered. Consequently, $H$ value is 0 for an analyzed population composed of a single species ($P_i = 1$), and increases with the diversity of species. The maximal value is obtained when a single isolate is present for each species encountered.

### 2.4. C. perfringens toxin genes amplification

Phospholipase C gene (*cpa*) was amplified as described previously (Titball et al., 1989). Primers cpa-F (ATA-GAT-ACT-CCA-TAT-CAT-CCT-GCT) and cpa-R (AAG-TTA-CCT-TTG-CTG-CAT-AAT-CCC) were used at a final concentration of 0.3 μM, in a mix containing 1× buffer, dNTP 0.2 mM, $MgCl_2$ 1.5 mM and 2 units of *Taq* DNA polymerase (GoldStar®, Eurogentec, Angers, France) for 25 μL. For each assay, 5 μL of DNA was used as template. PCR was performed using the following program in the thermocycler (GeneAmp PCR system 9700, Applied Biosystem): initial denaturation at 94 °C 5 min; 30 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 30 s; followed by a final extension step at 72 °C for 10 min. A 281-pb amplicon was revealed with the GelDoc XR imaging apparatus (Bio-Rad) after 1% agarose in TAE electrophoretic separation in presence of ethidium bromide.

Enterotoxin gene (*cpe*) was amplified according to Fach and Popoff (1997) and to Miki, Miyamoto, Kaneko-Hirano, Fujiuchi, and Akimoto (2008). Primers P145 (GAA-AGA-TCT-GTA-TCT-ACA-ACT-GCT-GGT-CC) and P146 (GCT-GGC-TAA-GAT-TCT-ATA-TTT-TTG-TCC-AGT) were used at a final concentration of 0.5 μM (Fach & Popoff, 1997). Primers cpe-F3 (ACA-TCT-GCA-GAT-AGC-TTA-GGA-AT) and cpe-R3 (CCA-GTA-GCT-GTA-ATT-GTT-AAG-TGT) were used at a final concentration of 1.0 μM (Miki et al., 2008). For both assays, the PCR mixture contained 10 μL of template DNA in a final volume of 45 μL. PCR mixture was composed of 1× buffer, dNTP 0.2 mM, $MgCl_2$ 2.5 mM and 3 units of *Taq* DNA polymerase (GoldStar®, Eurogentec, Angers, France) for 45 μL. The PCR program remained unchanged. Amplicons of 425 bp and 248 bp were expected respectively with primers P145/P146 and cpe-F3/cpe-R3.

Five isolates, kindly provided by Dr Messelhaüsser (Bavarian Health and Food Safety Authority, Germany) were used as positive controls: E723 and E728, isolated from human stool sample, 1312, isolated from backed potatoes (foodborne outbreak), 6682/1 isolated from Chili con carne (foodborne outbreak), and L93 isolated from vegetables. These isolates were all positive for *cpa* and *cpe* genes PCR detection, as tested in this study.

### 2.5. Growth experiments in foie gras

Sterilized foie gras was used for growth experiments to avoid any competition between microorganisms. Three different and independent foie gras batches were used for each strain. For each assay, 15 g-slices of foie gras were used. Slice surface was inoculated by patches of 0.3 mL of a 48 h-culture cold-adapted at 8 °C for 48 h, and diluted to $10^5$–$10^4$ cfu/mL. One sterile plastic flask containing one inoculated foie gras slice was prepared for each sampling time. Non-inoculated controls were prepared for each foie gras batches. Sterile flasks with slightly unscrewed plastic caps were placed into an anaerobic jar at 8 °C. Separated jars were planned for each sampling time. At each sampling time, foie gras was decimally diluted in tryptone–salt broth prior enumeration.

### 2.6. Heat resistance

Heat resistance of spores under standardized conditions was assayed during a time-course experiment. Spore suspension in phosphate buffer pH 7 was poured into 100 μL capillary glass tubes (Ringcaps® Duran®). The tubes were immersed into a thermostated oil bath. After defined thermal treatment times, tubes were cooled