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Diversity of the dominant bacterial species on sliced cooked pork products at expiration date in the Belgian retail

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ABSTRACT

Pork-based cooked products, such as cooked hams, are economically valuable foods that are vulnerable to bacterial spoilage, even when applying cooling and modified atmosphere packaging (MAP). Besides a common presence of *Brochothrix thermosphacta*, their microbiota are usually dominated by lactic acid bacteria (LAB). Yet, the exact LAB species diversity can differ considerably among products. In this study, 42 sliced cooked pork samples were acquired from three different Belgian supermarkets to map their bacterial heterogeneity. The community compositions of the dominant bacterial species were established by analysing a total of 702 isolates from selective agar media by $(GTG)_5$-PCR fingerprinting followed by gene sequencing. Most of the isolates belonged to the genera *Carnobacterium*, *Lactobacillus*, and *Leuconostoc*, with *Leuconostoc carnosum* and *Leuconostoc gelidum* subsp. *gelidum* being the most dominant members. The diversity of the dominant bacterial species varied when comparing samples from different production facilities and, in some cases, even within the same product types. Although LAB consistently dominated the microbiota of sliced cooked pork products in the Belgian market, results indicated that bacterial diversity needs to be addressed on the level of product composition and batch variation. Dedicated studies will be needed to substantiate potential links between such variability and microbial composition. For instance, the fact that higher levels of lactobacilli were associated with the presence of potassium lactate (E326) may be suggestive of selective pressure but needs to be validated, as this finding referred to a single product only.

1. Introduction

Cooked pork products, such as cooked hams, are food products of major economic importance, as they make up about one fourth of the delicatessen sold in Europe (Casiraghi et al., 2007). As these products are very susceptible to bacterial spoilage, several preservation techniques are used to increase their shelf-life. These techniques include storage at low temperature under modified atmosphere packaging (MAP) and the addition of additives (E compounds), such as curing salts, lactate salts, and ascorbate (McMillin, 2008; Thippareddi and Phebus, 2007; Vasilopoulos et al., 2015). More innovative routes explore the use of bioprotective cultures of lactic acid bacteria (LAB) for meat and fish preservation (Vermeiren et al., 2006; Vasilopoulos et al., 2010b, 2015; Cifuentes Bachmann and Leroy, 2015). Yet, these may still encounter legislative bottlenecks and generally seem to require a better understanding of the dynamics of the bacterial communities involved as well as their metabolism within the product during storage in retail.

In general, the bacterial composition of the final cooked pork product depends on the raw materials used and the technology applied. In a cold chain, for instance, the growth of mesophilic and thermophilic bacteria is reduced, while psychrotolerant and psychrophilic bacteria are selected for (Pothakos et al., 2014, 2015). The use of gas mixtures of N_2 and CO_2 in MAP products favours microbiota that withstand anaerobic conditions, usually consisting of LAB species and *Brochothrix thermosphacta* (Björkroth et al., 2005; McMillin, 2008; Samelis et al., 2000; Vasilopoulos et al., 2015). It excludes putrefying species belonging to the genera *Acinetobacter*, *Moraxella*, *Pseudomonas*, and *Psychrobacter* that are typical for aerobic storage. Moreover, variations in salt concentration, phosphate levels, and the general use of additives can affect the bacterial ecology of meat (Gögüs et al., 2007; Drosinos et al., 2006; Devlieghere et al., 2009; Nieminen et al., 2012). This is

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especially of importance since E compounds are badly perceived by many consumers, even though they often lead to technological and preservative benefits, which results in the adoption of specific criteria and labels by the meat processing industry (Resconi et al., 2016). In Belgium, for instance, the "Meesterlycke" label has been introduced for cooked hams to reassure consumers, requiring that there are no chemical additives supplemented to the meat and that the concentration of sodium chloride remains below 2% (m/m) (VLAM, 2016). These products are therefore particularly prone to spoilage by bacteria (Vasilopoulos et al., 2008). Spoilage usually manifests itself as discolouration or as the production of slime, gas, acidity, and/or off-odour compounds (Samelis et al., 1998; Drosinos et al., 2006; Iacumin et al., 2014; Remenant et al., 2015; Vasilopoulos et al., 2015).

In this study, a market survey of the dominant bacterial communities within cured and sliced cooked pork products in the Belgian retail was carried out at their expiration date. The aim was to obtain improved insights into the variability of the bacterial cooked pork ecosystem of products commonly present in the market. Such information is needed as to develop better spoilage control mechanisms, for instance in view of the development of potential bioprotective cultures to deal with a specific microbiota (Vasilopoulos et al., 2010b, 2015).

2. Materials and methods

2.1. Sample acquisition

Forty-two samples of cured and sliced cooked pork products stored under MAP (unspecified gas mixture and packaging material) were bought in three different Belgian supermarket chains between October 2014 and June 2015 (Table S1, Supplemental material). A total of 25 sliced cooked pork product samples contained only the technological minimum of additives, namely sodium nitrite (E250) and sodium ascorbate (E301). The remaining samples also had one or more of the following additives: caramel colour (E150; 1 samples), carotenoid (E160; 1 samples), sodium acetate (E262; 2 samples), potassium lactate (E326; 4 samples), carrageenan (E407; 8 samples), pyrophosphate (E450; 7 samples), sodium triphosphate (E451; 13 samples), polyphosphate (E452; 4 samples), potassium chloride (E508; 4 samples), and/or disodium ribonucleotides (E635; 3 samples). These samples originated from nine different production facilities, corresponding with sixteen different product types (Table 1). Product types were defined as products carrying the same commercial label and branding, thus also being characterized by identical ingredient composition and additives (E compounds) and coming from the same producer. After acquisition, the samples were stored at 4 °C until their expiration date.

2.2. General analysis of the samples

Prior to the microbiological analysis, samples were checked for visual signs of spoilage, in particular discoloration and slime formation. After sampling, the products were subjected to a pH measurement (pH electrode SenTix Sp; WTW, Weilheim, Germany). For analysis of dominant bacterial communities, 10–15 g of meat of each cooked pork product sample was brought into a stomacher bag, diluted 10 times using a peptone physiological solution [0.85% (m/v) NaCl and 0.1% (m/v) peptone in ultrapure water], and mechanically treated for 90 s at medium speed, using a Stomacher 400 (Seward, Worthington, UK). Subsequently, dilution series were made in peptone physiological solution and subjected to pour plating. The overall bacterial biodiversity was measured on plate count agar, acting as a universal medium (PCA; Oxoid, Basingstoke, Hampshire, UK). The specific dominant microbiota of lactic acid bacteria was measured on de Man-Rogosa-Sharpe agar (MRS; Oxoid) adjusted to pH 5.9, as well as on modified MRS (mMRS) agar for a better recovery of the subfraction of carnobacteria (Vasilopoulos et al., 2010a), and on M17 agar (Oxoid) targeting lactococci. For the elaboration of mMRS from single ingredients, MRS medium was adjusted by omitting acetate and increasing the pH to 8.6. After overlaying with soft agar (1.2%, m/m), the plates were incubated at 22 °C for five days.

2.3. Isolation and identification of bacterial species

Between 10 and 30% of the colonies were picked up from the highest dilution of each agar medium (corresponding with 5–25 isolates) and transferred into brain heart infusion (BHI) medium (Oxoid). After incubation at 22 °C for 24–48 h, the obtained cultures served to generate cell pellets by centrifugation at 13,793×*g* for 5 min (Biofuge 13; Heraeus, Osterode, Germany). Therefore, for genomic DNA extraction, the cell pellets were washed using Tris-EDTA-Sucrose (TES) buffer (0.2 M sucrose, 0.001 M EDTA, and 0.05 M Tris-base; pH 8.0). After adding 200 μl of Tris-EDTA-Triton-X-100 (TET) buffer [0.02 M Tris-HCl, 0.002 M EDTA, and 1% (m/ml) Triton X-100; pH 8.0) containing 4.0 mg of lysozyme (Merck, Darmstadt, Germany) and 100 U of mutanolysin (Sigma-Aldrich, St. Louis, MO, USA), The cell pellets were incubated at 37 °C for 60 min. Afterwards, mixtures were treated with 25 μl of proteinase K (29.0 mg/ml) (Macherey-Nagel, Düren, Germany) at 56 °C for 60 min. Genomic DNA extraction and purification were completed using the NucleoSpin®96 tissue kit (Macherey-Nagel), according to the manufacturer's instructions. Next, DNA concentrations were measured using a Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, MA, USA). $(GTG)_5$-PCR was performed using a denaturation step at 95 °C for 5 min, followed by 30 cycles of 1-min denaturation at 94 °C, hybridisation at 40 °C for 1 min, extension at 65 °C for 8 min, and a final step of 10 min at 65 °C (Vasilopoulos et al., 2008). PCR amplicons were run on agarose gels (constant voltage, 60 V, 960 min) and stained with ethidium bromide for 20 min. Classification and identification of the isolates was based on numerical cluster analysis of $(GTG)_5$-PCR fingerprints of genomic DNA, using Bionumerics software (v. 5.10; Applied Maths, Sint-Martens-Latem, Belgium), as outlined previously (Janssens et al., 2012). For numerical cluster analysis, the Pearson correlation coefficient and the UPGMA (unweighted pair group method with arithmetic mean) method were applied. PCR and electrophoresis reproducibility were routinely checked using molecular markers and the strain *Lb. plantarum* LMG 6709^T for a positive control. The identity of representative isolates from each $(GTG)_5$-PCR fingerprint cluster was confirmed via sequencing of the 16S rRNA gene from genomic DNA, as described previously (Vasilopoulos et al., 2008). Additional sequencing of the *pheS* gene was performed to further confirm the identification of the strains (Snauwaert et al., 2013; De Bruyne et al., 2007). The gene sequencing results were processed using the BioEdit Sequence Alignment Editor (v. 7.2.5.0; Ibis Biosciences, Carlsbad, CA, USA) and identities were assigned with the basic local alignment search tool (BLAST) and NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4. Statistical analysis

After processing in Microsoft Excel, the data were transferred to IBM SPSS Statistics (v. 20; IBM Corporation, Armonk, NY, USA) for a principal component analysis, using the bacterial cell counts from the four cultivation media (divided along eight classes ranging from 5.5 to 9.5 log (cfu g^{-1}) with a 0.5 interval), the prevalence of the different bacterial genera, the pH values (divided along eight