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Influence of lactate and acetate removal on the microbiota of French fresh pork sausages

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ABSTRACT

The microbiota of fresh French pork sausages were characterised in five batches of comminuted pork meat that were equally divided into two formulations either containing the acid-based preservatives lactate and acetate, or no preservatives. Conventional microbiological analysis and high-throughput 16S rDNA amplicon sequencing methods were performed on meat batches packed under modified atmosphere (70% oxygen and 30% carbon dioxide) during chilled storage. In addition, meat pH and colour, and gas composition of the packages were monitored until the end of the shelf-life. During storage, the population of mesophilic and lactic acid bacteria increased from 4 log CFU/g to 8 log CFU/g after 15 days of chilled storage, both with and without preservatives. Despite similar changes of the physical and chemical parameters, such as pH and package gas composition, spoilage was delayed in the meat containing the preservatives, suggesting that lactate and acetate are effective against spoilage. Metagenetic analysis showed that at the end of the shelf-life, the species distribution differed between both the formulations and the batches. Lactic acid bacteria were shown to dominate both with and without preservatives; however, samples containing no preservatives were characterised by the presence of an increased population of *Brochothrix* spp. and *Pseudomonas* spp. whereas, *Leuconostoc mesenteroides/pseudomesenteroides* and *Lactobacillus curvatus/graminis* were more abundant in the meat with preservatives.

1. Introduction

In the meat industry, professionals are required to ensure product safety by controlling contamination, development of food-borne pathogens, and any subsequent toxin production. Moreover, they have to ensure product stability by controlling microorganisms that are known to cause spoilage. In fresh meat, there are many sources of microbial contamination including, the animal and the carcasses (Chaillou et al., 2015; De Filippis et al., 2013) as well as from the environment in which the food processing is conducted (Chaillou et al., 2015). Fresh pork sausages are highly perishable products with a shelf-life generally below 15 days, and spoilage microorganisms are likely to be the major factor determining this shelf-life. The main techniques used to control the quality of fresh sausages and to extend their shelf-life include the use of salting, modified atmosphere packaging, storage under chilled conditions, and the addition of food preservatives such as lactate and acetate. Lactate is known as a preservative agent with antimicrobial properties that enable an extension of the meat product shelf-life (Bradley et al., 2011; Brewer et al., 1991; Houtsma et al., 1993; Lamkey et al., 1991; Tan and Shelef, 2002). Acetate is another organic acid, also known to inhibit bacterial growth (Juneja and Thippareddi, 2004; Lamas et al., 2016). In Europe, both lactate and acetate are listed on the product pack label as chemical preservatives (Regulation (EC) No 1333/2008); however, currently there is an increasing consumer demand for safe products with less salt and preservatives. Though, the removal of food preservatives such as lactate and acetate from fresh pork sausages is likely to result in spoilage of the meat and increased safety concerns.

Studies have been conducted to determine the impact of preservatives on the quality of meat products by enumerating the bacterial groups present in the meat, with conventional culturing methods (Cegielska-Radziejewska and Pikul, 2004; Crist et al., 2014; Deumier and Collignan, 2003). These studies aimed to determine the growth kinetics of the total mesophilic or psychrophilic bacteria and/or lactic acid bacteria during chilled storage rather than investigating the diversity and structure of the microbiota as result of addition of different preservatives. The emerging metagenetic approach of high-throughput 16S rDNA amplicon sequencing has recently been used to characterise bacterial communities in various foods, especially fermented foods (Alessandria et al., 2016; Delcenserie et al., 2014; Jung et al., 2011; Nam et al., 2012; Ramezani et al., 2017). It has proven to be an efficient, sensitive, and fast technique to identify potential sources of

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contamination, and to explore the evolution of the bacterial ecosystem along the processing steps and storage duration (De Filippis et al., 2013; Ercolini, 2013). However, few studies have characterised the microbiota of meat products (Chaillou et al., 2015; De Filippis et al., 2013; Delhalle et al., 2016; Ercolini et al., 2011; Hultman et al., 2015; Nieminen et al., 2012; Piotrowska-Cyplik et al., 2017; Wang et al., 2016) and even less have investigated the influence of the product formulation on the bacterial communities present in the meat. The influence of the preservatives on the microbiota in meat has only been studied in the following studies: during the storage at 4 °C of fresh pork sausages produced with different levels of organic acids (sodium lactate and sodium diacetate), when reducing the salt content in raw pork meat sausages and on marinated and unmarinated broiler fillet strips after 13 days of storage at 6 °C, respectively (Benson et al., 2014; Fougy et al., 2016; Nieminen et al., 2012).

Spoilage remains a complex process, which depends not only on any initial contamination, but also on conditions encountered during food processing and storage (Hultman et al., 2015). Both of these factors contribute to the microbiota that is established during the product shelf-life. Considering the low number of studies investigating the effect of the formulation on the microbiota in meat products, and the high variability in the processes used and the influence of initial contaminations, studying the impact of food preservatives on the microbiota in fresh pork sausages remains of interest. Investigating the effect of preservatives on meat products will further our understanding of food ecosystem dynamics and ultimately identify control strategies to limit spoilage.

In this study, two formulations of French pork sausages were studied, one containing sodium acetate and potassium lactate, used as preservatives, and another without any preservatives. The bacterial ecosystem and the changes of physical and chemical parameters of the fresh pork sausages, conditioned under modified-atmosphere packaging during chilled storage, were investigated. The aim of the study was to compare the microbiota in both formulations in order to identify the bacteria present following preservative removal, to attempt to correlate their presence with accelerated spoilage.

2. Materials and methods

2.1. Fresh pork meat sausages

Sausages were prepared by a French local meat processing company. Five batches of comminuted pork meat composed of 69–73% shoulder and 21–21.5% fat, were produced over five different days of production. Each batch was then equally divided into two parts. The first part was supplemented with a 'clean' mix, composed of 3% water and 3% of a mix containing salt, natural flavours, glucose, spices, and silicium dioxide. The second part was supplemented with a 'preservative' mix. It was composed of 5% water, 1.5% potassium lactate and 3% of a mix containing salt, glucose, sodium acetate, spices, sodium erythorbate, cochineal, curcumin, and natural flavours. Consequently, from each day of production (A, B, C, D and E) two sausage formulations were produced CLN and PRS, corresponding to the addition of the 'clean' mix or the 'preservative' mix, respectively. The initial microbial contamination of the comminuted meat used for both formulations was the same for each day of production.

Sausages were packaged in groups of six in crystalline polyethylene terephthalate trays (CPET), sealed with a plastic film (polyethylene/ethylene-vinyl alcohol copolymer resin (EVOH)/Polyethylene (PE)/Polypropylene (PP)) and stored under modified atmosphere packaging (MAP) (70% O_2 and 30% CO_2). Sausage trays were received one day after production and thereafter stored at 4 °C for 8 days, then at 8 °C for 11 days, according to the storage protocol established in France to determine the shelf life of chilled perishable and highly perishable food (AFNOR, 2010). The use-by-date established by the manufacturer was 12 days at 4 °C.

2.2. Sampling

One hundred sausage trays per formulation were used in this study. After 1, 12, 15, and 19 days of chilled storage, 5 sausage trays were collected for each batch and formulation. They were adequately divided between the analyses to be performed; physical and chemical, microbiological and pyrosequencing.

2.3. Microbiological analysis

2.3.1. Conventional culturing methods

Culturing methods were performed on day-1, day-12, and day-15, for both formulations on batches C, D, and E. A total of 20 g of meat was sampled, blended, and diluted in a sterile stomacher bag with 80 mL of peptone water solution (AES, France) containing 1% tween-80 (Sigma Aldrich, France). After 1 min of homogenisation and 20 min at ambient temperature to allow the recovery of the bacteria, meat suspensions were serially diluted in tryptone salt buffer (1 g/L tryptone, Biokar, France; 8.5 g/L sodium chlorure, Panréac, France). All bacterial counts were performed in duplicate. Five different bacterial groups were counted in this study; the total viable count (TVC) on plate count agar (PCA) (Becton Dickinson, France) incubated at 30 °C for 48 h, lactic acid bacteria (LAB) on De Man Rogosa Sharpe (MRS) agar (Biokar, France) incubated anaerobically at 25 °C for 72 h, *Enterobacteriaceae* on violet red bile glucose (VRBG) agar (Biokar, France) incubated at 30 °C for 24 h, *Pseudomonas* spp. on cetrimide, fucidin, cephalosporin (CFC) agar base (Biokar, France) supplemented with the CFC supplement (Biokar, France) incubated at 25 °C for 48 h, and *Brochothrix* spp. on streptomycin sulphate thallous acetate agar (STAA) base (Oxoid, France) supplemented with the STAA supplement (Oxoid, France), incubated at 25 °C for 48 h. All plates were inoculated by the spread-plate method except VRBG plates, which were inoculated by pour-plate method.

2.3.2. rDNA amplicon pyrosequencing

rDNA amplicon pyrosequencing was performed on samples 12 days after production for all batches and formulations. 'Twelve days' represents the current use-by-date of the sausages in the PRS formulation. Furthermore, spoilage is supposed to occur around this day or after, rDNA amplicon pyrosequencing should enable to visualise the bacterial communities with an adequate sequencing depth to ensure a valid comparison between formulations. Thus, rDNA amplicon pyrosequencing was not performed at the beginning of storage because the initial contamination was too low in some batches, resulting in low DNA extraction yields. Moreover, the same comminuted meat per batch was used whether preservatives were added or not.

DNA extractions were performed on two 4.5 mL samples of 1/5 dilutions of the meat suspensions in peptone water, as described previously in Section 2.3.1. Suspensions were placed on filters of the NucleoSpin Plant II kit (Macherey-Nagel, France) and centrifuged in a 5810R Eppendorf centrifuge, at 8000 rpm at 4 °C for 5 min for the first 4.5 mL, then at 10 000 rpm at 4 °C for 5 min for the second 4.5 mL. The supernatants were removed and extractions were performed on the pellets with the PowerFood Microbial DNA isolation kit (Mo Bio Laboratories, USA) according to the manufacturer's instructions. DNA was eluted in 100 µL of elution buffer and stored at −20 °C until required.

Amplification of the 16S rDNA V1-V3 region was performed using 27F and 534R primers (Lane, 1991) fused with a Matrice Identity (MID) sequence specific to the sample, for sorting at the end of the pyrosequencing, and an adaptor required for hybridisation of the PCR product on the microbead during pyrosequençing. The PCR mixture (50 µL final volume) contained 1X Taq buffer (10 mM Tris-HCl, pH 8.85, 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.6 µM of each primer and 2.5 U Pwo DNA polymerase (Roche, France), and 5 µL of DNA extract. PCR amplifications were performed in a T100 Thermal cycler (Bio-Rad) using the following protocol: initial denaturation 94 °C for 2 min, followed by 25 cycles of denaturation (94 °C