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A method to isolate bacterial communities and characterize ecosystems from food products: Validation and utilization in as a reproducible chicken meat model

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ABSTRACT

Influenced by production and storage processes and by seasonal changes the diversity of meat products microbiota can be very variable. Because microbiotas influence meat quality and safety, characterizing and understanding their dynamics during processing and storage is important for proposing innovative and efficient storage conditions. Challenge tests are usually performed using meat from the same batch, inoculated at high levels with one or few strains. Such experiments do not reflect the true microbial situation, and the global ecosystem is not taken into account. Our purpose was to constitute live stocks of chicken meat microbiotas to create standard and reproducible ecosystems. We searched for the best method to collect contaminating bacterial communities from chicken cuts to store as frozen aliquots. We tested several methods to extract DNA of these stored communities for subsequent PCR amplification. We determined the best moment to collect bacteria in sufficient amounts during the product shelf life. Results showed that the rinsing method associated to the use of Mobio DNA extraction kit was the most reliable method to collect bacteria and obtain DNA for subsequent PCR amplification. Then, 23 different chicken meat microbiotas were collected using this procedure. Microbiota aliquots were stored at −80 °C without important loss of viability. Their characterization by cultural methods confirmed the large variability (richness and abundance) of bacterial communities present on chicken cuts. Four of these bacterial communities were used to estimate their ability to regrow on meat matrices. Challenge tests performed on sterile matrices showed that these microbiotas were successfully inoculated and could overgrow the natural microbiota of chicken meat. They can therefore be used for performing reproducible challenge tests mimicking a true meat ecosystem and enabling the possibility to test the influence of various processing or storage conditions on complex meat matrices.

1. Introduction

Microbial diversity is shaping the ecology of very diverse ecosystems. For example, bacteria are known to be a major part of geochemical cycles in natural environment. Studying the microbial diversity and interactions of bacteria with the support and the other organisms is always a challenge due to the extreme variability which can occur between samples. In meat agro-food industry, contaminating bacteria originate from animal microbiota (feces, hide, skin, or feather), from production plant environment (air, equipment, surfaces) and from human manipulators (Chaillou et al., 2015). Therefore, a large diversity of species can be hosted by meat products. After initial contamination of carcasses or cuts, processing steps and storage conditions like temperature and the atmosphere used for packaging, shape the evolution of this microbiota. The microbial diversity and its dynamics during food production can influence the product shelf life and safety if spoilage bacteria are favored and pathogenic bacteria present and able to grow.

In poultry meat, the total viable counts reported in the literature and expressed as colony forming units per gram (CFU/g) ranges from 6.5 to 9 log depending on authors, and on storage conditions and poultry cuts (Björkroth, 2005; Balamatsia et al., 2007; Chouliara et al., 2007; Zhang et al., 2012; Al-Nehlawi et al., 2013; Capita et al., 2013). This suggests that a great quantitative variability of bacterial contamination hosted by poultry meat exists. *Pseudomonas* sp., *Enterobacteriaceae*, *Brochothrix thermosphacta*, and lactic acid bacteria such as *Carnobacterium* sp. and lactotobacilli are among the most often bacterial contaminants reported by authors. A large majority of the published results are focused on pathogenic bacteria whereas spoilage microorganisms were rarely investigated. Indeed, *Salmonella* and *Campylobacter* prevalence, or characteristics of *Staphylococcus aureus* isolates from poultry cuts have been reported from several countries (see as examples Atanassova and Ring, 1999; Capita et al., 2001, 2002, 2007). In addition, only few studies dealing with the whole microbiota of poultry meat have been reported (del Río et al., 2007; Hinton and Ingram, 2003; Nieminen et al., 2012). Many articles focused only one bacterial species and did not consider

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the natural bacterial contaminants, despite their impact on the bacterial dynamics. For instance on pork meat, the conclusions drawn by using *Salmonella* growth predictive models were different when sterile or naturally contaminated meats were used, the natural microbiota of meat reducing *Salmonella* growth (Møller et al., 2013). This example shows the importance to consider food matrices as a global ecosystem hosting complex microbial communities (Fleet, 1999).

Several studies aiming at understanding the mechanisms of bacterial adaptation to food environment have been reported. In general, the approaches used are based on challenge tests in which bacteria (commonly one or a few strains) are inoculated at empirical levels, which do not always reflect the conditions that occur in commercialized and consumed products. As an example, the effect of modified atmosphere packaging on the growth of *Campylobacter* was studied on chicken breast fillet by inoculating meat at 10^4 to 10^5 CFU/g with a five-strain cocktail (Meredith et al., 2014). Although informative the results obtained in such conditions, do not reflect the real situation of the products that can be proposed on the market as the concentration of *Campylobacter* in naturally contaminated products is difficult to estimate (Rohonczy et al., 2013). Indeed, most often only prevalence of *Campylobacter* is reported (see Economou et al., 2015 as example) and only few reports about the contamination level are available, as it varies along the food chain and is batch-dependent (Gruntar et al., 2015).

Poultry meat samples constitute very heterogeneous matrices depending on the type of cuts. The unavoidable bacterial contamination occurs mostly at the surface and on the skin of the cuts during the different steps of the slaughtering process (Luber, 2009). The poultry meat worldwide production is in constant increase each year reaching 106.8 million tons in 2013. In connection with the human population growth, the needs for meat production also increase especially in developing countries. According to the FAO, increased consumption is mainly due to an attractive price-quality ratio and to health and nutrition benefits of poultry meat. On the other hand, chicken meat attractivity increases because producers develop retails and ready-to-eat products, fast and easy to prepare, fitting with to consumers demand. It is therefore necessary to guaranty the safety of poultry meat to face this increasing demand.

The effects of different treatments have been studied in order to develop strategies for fighting human pathogens or spoilage species. Among those the use of modified atmosphere packaging, alone (Al-Nehlawi et al., 2013; Meredith et al., 2014) or combined to protective cultures (Melero et al., 2012) or essential oils (Chouliara et al., 2007) as well as decontamination with various chemicals (Okolocha and Ellerbroek, 2005; del Río et al., 2007; Alonso-Hernando et al., 2012; Capita et al., 2013) are the most documented. The effects of other treatments such as irradiation (Szczawińska et al., 1991) or marinades (Nieminen et al., 2012) have also been described. To overcome variability, microbiologists usually inoculate food or matrices from one batch in order to obtain reproducible matrices. In microbial ecology studies aiming to elucidate bacterial interactions, with the food matrix and/or other micro-organisms, the challenge is i) to define reproducible and reliable experimental conditions to lead to biological interpretation, or ii) to multiply sampling or experiments to obtain statistical significance of the results. In the present study we designed a method to collect poultry meat bacterial communities in order to develop an accurate model useful to reproducibly investigate the effect of various meat processing and storage conditions on the evolution of meat microbiota. In France chicken legs are a popular meal and are often sold as portions of 2, 4, 6 or more legs packaged under various modified atmospheres. In addition, a large choice of meat is proposed, issued from various farming practices (including organic, free-range, "label rouge" farming), and performed on various genetic backgrounds (white, yellow and black races). We took into account this large diversity of producing conditions in our sampling procedure and collected microbiota from chicken meat to constitute a livestock that could be characterized and used to re inoculate fresh matrices to create a standard ecosystem.

2. Materials and methods

2.1. Chicken meat samples

Chicken cuts (portions of 2 legs or 1 kg - *i.e.* 4–6 - breast fillets) stored under modified atmosphere were collected from local supermarkets on the day of arrival, *i.e.* 1–2 days after slaughtering, and stored at 4 °C until experiments. Gas composition of the meat packages was measured just before collecting bacteria as described by Melero et al. (2012) using a digital O_2/CO_2 analyzer (Oxybaby, WITT Gasetechnik GmbH & Co. KG, Germany).

For the constitution of life stocks representing diverse bacterial communities naturally present on poultry meat 23 packs of two chicken legs (coined here A to W) from various origins and labels were used. The characteristics of the 23 samples are summarized in Table 1. After rinsing one leg for 5 min in 200 mL TS, bacteria were collected by centrifugation, the pellet was resuspended in 85 mL of TS and 1 mL-aliquots were stored at −80 °C for further studies. Bacteria were enumerated before and after various freezing periods at −80 °C (1 to 28 weeks depending on batches).

2.2. Bacteria collecting

The experimental design to set up a reliable method for collecting and store the bacterial communities from meat samples is summarized Fig. 1. Four different treatments were tested to recover bacteria from meat (stomaching, rinsing, swabbing, and scrapping). Collected bacteria were resuspended in sterile TS then stored at −80 °C as 1 mL aliquots with 15% (v/v) glycerol and the efficiency of each treatment was estimated by CFU counting at each step (Fig. 1).

2.2.1. Stomaching

Fifty grams of meat were aseptically transferred into a sterile stomacher bag, with 200 mL TS (8.5 g/L NaCl, 1 g/L tryptone in distilled water) containing 1% Tween 80. Meat samples were then homogenized for 2 min in a stomacher (Masticator, IUL Instruments, England). The homogenate was filtered through the bag filter and centrifuged through a filter (F) or a column (C) from Nucleospin Plant II Midi kit (Macherey Nagel, EURL, France) at 8000 ×*g* during 10 min at room temperature. These filters bind cell fragments whereas columns bind eukaryote DNA from the matrix. Unlysed bacteria were therefore collected in the pellet and resuspended into 3.3 mL TS. Alternatively, 30 mL of blended mixture were filtered by gravity through a sterile paper filter or used for 2 successive centrifugation steps a low gravity to remove food residues: 30 mL were first centrifuged at 100 ×*g*, 3 min at room temperature and 25 mL of supernatant were subsequently centrifuged at 500 ×*g* for 5 min. Then 20 mL of filtrate or supernatant were centrifuged at 3000 ×*g* 20 min at 4 °C and the bacterial pellet was resuspended into 3.3 mL TS.

2.2.2. Rinsing

A whole portion of meat was added with 200 mL TS into a sterile stomacher bag. Alternatively TS containing 1% Tween 80 or peptone water (peptone 10 g/L, sodium chloride 5 g/L, disodium phosphate 3.56 g/L, potassium dihydrogen phosphate 1.5 g/L, pH 7.2 at 25 °C) were tested. Hand-agitation was performed during 30 s to 5 min. The liquid was filtered through the bag filter, centrifuged at 4000 ×*g* for 20 min at 4 °C then the bacterial pellet was suspended into 100 mL TS.

2.2.3. Swabbing

A 5 cm × 5 cm zone on chicken skin was swabbed. The swab (Copan Diagnostic 155C, Italy) was vortexed with 5 mL TS containing 1% Tween