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Quantification and efficiency of *Lactobacillus sakei* strain mixtures used as protective cultures in ground beef

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ABSTRACT

Lactobacillus sakei is a lactic acid bacterium, naturally associated with long term storage of fresh meat at low temperature. Here we investigated the effect, on the evolution of pathogenic and spoilage microorganisms in ground beef, of *L. sakei* cocktails used as bioprotective cultures. We selectively developed a real time quantitative PCR method, allowing the quantification of individual *L. sakei* strains inoculated in ground meat with specific probes. Six cocktails of three strains were tested to evaluate their effect on the growth of *Salmonella enterica* Typhimurium, *Escherichia coli* O157:H7 and *Brochothrix thermosphacta* at 4 °C and 8 °C, under vacuum or modified atmosphere packaging. Using plating methods to quantify the different bacterial species, one cocktail showed an effect against *S.* Typhimurium and *E. coli* under given conditions. Real time quantitative PCR showed that the three inoculated *L. sakei* strains had a different growth pattern, and that the association of these three strains indeed impaired growth of *S.* Typhimurium and *E. coli*.

1. Introduction

Meat products are highly perishable, due to bacterial spoilage that can alter their color, texture or odor. Among spoilage organisms, *Brochothrix thermosphacta* is able to grow at low storage temperature used for meat preservation and can alter meat, including beef (Dainty & Hibbard, 1980; Gribble & Brightwell, 2013). In addition, these products can host pathogens. The recent food borne outbreaks which have taken place over the ground beef, especially with *Escherichia coli* O157:H7, state the need for solutions to ensure ground meat safety. Consumers have dramatically changed their life style but they still have high demands regarding beef meat including (i) safe beef and beef products with upgraded sensory quality (ii) increased functional and nutritional properties and (iii) traditional, wholesome image. They also exert strong pressure to reduce additives, minimizing processing and intervention (Gould, 1996). There is thus a need to take into account all these parameters, even if some appear contradictory.

Biopreservation has gained increasing attention as mean of naturally controlling the shelf life and safety of meat products. The application of protective cultures to ensure hygienic quality is a promising tool although, as pointed out by Holzapfel, Geisen, and Schillinger (1995), it should be considered only as an additional measure to good manufacturing, processing, storage, and distribution practices. Some microorganisms commonly associated with meats have proved to be antagonistic towards pathogenic and spoilage bacteria. In particular, lactic acid bacteria (LAB) have a major potential use in biopreservation because they are safe for human consumption and are the prevalent microflora during storage in many foods. Among those, *Lactobacillus sakei*, commonly used for the fermentation of dry sausage has a long history of use in human food and therefore a GRAS (Generally Recognized As Safe) and QPS (Qualified Presumption of Safety) status (Bourdichon et al., 2012; EFSA, 2011). This bacterium has also been proposed to enhance non-fermented meat product microbial safety. Indeed, as a major component of the bacterial indigenous flora, it can inhibit the growth of *E. coli* in ground beef and, when re-inoculated in meat, several *L. sakei* meat isolates have been proven to be efficient as protective cultures against spoilage or pathogenic bacterial species (Bredholt, Nesbakken, & Holck, 1999, 2001; Katikou, Ambrosiadis, Georgantelis, Koidis, & Georgakis, 2005; Vold, Holck, Wasteson, & Nissen, 2000).

Besides these promising evidences, some bottlenecks do actually limit the development of such studies and their subsequent applications. Most of the studies aiming at testing the effect of putative protective cultures are based on a first screening in laboratory conditions, but antagonistic effects have often been described as abolished *in carnis* (Jones, Hussein, Zagorec, Brightwell, & Tagg, 2008; Jones, Zagorec, Brightwell, & Tagg, 2009; Katikou et al., 2005; Vermeiren, Devlieghere, & Debevere, 2006). As the protective effect against unwanted bacteria may result from various mechanisms, including bacteriocin or other antagonistic molecule production and competition for nutrients, one can hypothesize that mixtures of strains may be more efficient than single isolates. In particular, bacteriocins are often directed toward restricted

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groups of closely related bacteria, and the idea to use several strains to enlarge the diversity of targeted species looks realistic, but technically difficult to realize. Indeed, as bioprotective strains belong to indigenous species naturally occurring in meat, their monitoring by usual plating methods and CFU determination is rendered difficult. The natural resistance to various antibiotics or phenotypes easily identifiable on plates can sometimes be used to discriminate them among other strains or among the indigenous flora by plating methods (Vermeiren et al., 2006; Guilbaud, Zagorec, Chaillou, & Champomier-Vergès, 2012). Another alternative is to generate spontaneous antibiotic resistant mutants (Jones, Wilklund, Zagorec, & Tagg, 2010) but such mutants may harbor a different fitness than their wild type parent when grown in a natural environment (Chiaramonte, Blugeon, Chaillou, Langella, & Zagorec, 2009). Therefore, non-cultural methods may represent a nice alternative to detect and quantify specific bacterial species or even strains from complex meat ecosystems. PCR-DGGE has been successively used to distinguish bacterial species from ham treated with *L. sakei* protective cultures, but could not identify some spoilage bacteria and did not allow the quantification of the various species (Hu et al., 2008). Quantitative real time PCR (q-RT-PCR) is more promising (Martinez et al., 2011) as several species including *L. sakei* (Martin, Jofré, Garriga, Pla, & Aymerich, 2006) or spoilage organisms like *B. thermosphacta* and *Clostridium estertheticum* (Brightwell & Clemens, 2012; Gribble & Brightwell, 2013; Jones et al., 2009) have been successfully quantified in such a way.

The aim of the present study was to test the putative protective effect *L. sakei* strain mixtures against both pathogenic (*Salmonella* Typhimurium, *E. coli* O157:H7) and spoilage (*B. thermosphacta*) microorganisms in ground beef. As it has been previously shown that this species was genetically diverse and could be split into 10 different genomic groups (Chaillou et al., 2009), we aimed at pooling genetically different *L. sakei* strains in order to test the possibility of a wider protective efficiency and in a way that allowed monitoring each strain by using strain specific gene markers. Strains were therefore chosen without any *a priori* on their antagonistic affect, but rather on their genomic diversity.

2. Materials and methods

2.1. Bacterial strains, media, and culture conditions

L. sakei 18, 64, 112, 156, 160x1, 332, and G3 are meat isolates from the INRA collection (Chaillou et al., 2009). *L. sakei* CIP 105422 is a reference strain purchased from Pasteur Institute and *L. sakei* 23K is a plasmid cured strain from which the whole genome sequence has been determined (Chaillou et al., 2005). All these strains have previously been grouped into different genomic clusters, depending on the presence or absence of a set of reference genes (Chaillou et al., 2009). *L. sakei* strains were routinely grown in MRS broth (De Man, Rogosa, & Sharpe, 1960) at 30 °C. For meat inoculation, cultures were performed until a bacterial population of 10^8–10^9 CFU · ml^{-1} was reached (early stationary phase), using calibration curves OD_{600} *vs* CFU · ml^{-1} determined for each strain.

E. coli EDL933 *stx1/stx2* is an isogenic mutant of the reference strain EDL933, belonging to the serotype O157:H7 in which the genes and *stx2A-stx2B* were deleted and genes *stx1A-stx1B* were replaced by a kanamycin resistance cassette (Gobert et al., 2007). *B. thermosphacta* CIP103251T is a reference strain purchased from Pasteur Institute, and *Salmonella* Typhimurium ADIV S59 is a pork meat isolate from ADIV collection.

For meat inoculation, the three target strains were grown in Tryptone Soya broth (TS Oxoid) at 37 °C (*S.* Typhimurium and *E. coli*) or at 25 °C (*B. thermosphacta*) for the time needed to reach around 10^8 CFU · ml^{-1}, determined through spectrophotometric calibration curves OD_{600} *vs* CFU · ml^{-1} determined for each strain.

For chromosomal DNA extraction, bacteria were grown overnight in Tryptone Soja broth (*E. coli* and *S.* Typhimurium), Brain Heart Infusion (*B. thermosphacta*) or MRS (*L. sakei*).

From meat samples, total flora was quantified on Plate Count Agar (Oxoid-France) by colony counting after incubating plates 72 h at 25 °C. LAB population was enumerated after plating on MRS agar (Oxoid-France) and incubating at 30 °C for 72 h (ISO 15214, 1998). *B. thermosphacta* CFU was determined after plating on STA agar medium (Oxoid-France) and incubating at 25 °C for 48 h (NF V 04-505). *E. coli* and *S.* Typhimurium were enumerated after 24 h incubation at 37 °C on CT-SMAC (MacConkey Sorbitol agar, Oxoid-France, ISO 16654, 2001) and OSCM II (Oxoid *Salmonella* Chromogenic Medium II, Oxoid-France), respectively. Colonies of *E. coli* O157:H7 on CT-SMAC were identified using suitable phenotypical tests (glucose, lactose, and sorbitol fermentation; production of gas, H_2S, and indol; presence of urease and lysine decarboxylase activities; motility) and serological tests, according to Ewing (1986) and Toledo, Fontes, and Trabulsi (1982).

2.2. Ground beef challenge tests

2.2.1. Ground beef inoculation, packaging and storage condition

Serial dilutions of target bacteria (*S.* Typhimurium, *B. thermosphacta*, and *E. coli*) fresh cultures were prepared in buffered peptone water (CM0509, Oxoid, France), in order to inoculate between 10^2 and 10^4 · CFU · g^{-1} of minced meat, depending on the trials.

For *L. sakei* strains composing a cocktail, strains were grown separately, diluted in buffered peptone water and then pooled in order to obtain a mixture of equal concentrations of the three strains. Cocktails were inoculated at an initial population level ranging from 10^3 to 10^6 CFU · g^{-1} of minced meat.

The ground beef, containing 10% fat, used for the different trials was purchased directly after mincing in local slaughterhouses. Ground meat was immediately transported at 0–4 °C to the ADIV laboratory and frozen at −20 °C until use. Meat was defrosted at 4 °C for 16 h before inoculation. For each trial, the ground meat was divided into batches of 6 kg under aseptic conditions. One batch was used as non-inoculated control, and the other batches were artificially inoculated with the target strains and various *L. sakei* strain co-cultures. Controls without *L. sakei* inoculation were included. After homogenization, for a given batch, ground beef samples with an average weight of 125 g were prepared and then packaged under vacuum or modified atmosphere (70% O_2-30% CO_2). For each packaging type, half of the steaks were stored at 4 °C and the other half at 8 °C.

2.2.2. Microbial analysis of meat samples

For each trial, microbiological analyses were carried out according to three replicates (n = 3 steaks) just after inoculation (T0) at day 3 (middle of shelf-life, T3) and day 7 (shelf-life, T7) of storage under modified atmosphere, or at T0, T7 (middle of shelf-life) and T14 (shelf-life) for samples stored under vacuum-packaging.

To enumerate bacteria on plates, the sample preparation was conducted according to international standard ISO 7218 (2007) as follows: a 10-g ground beef aliquot was homogenized in 90 mL buffered peptone water in a stomacher bag using a stomacher machine (AES) for 60 s. Then, subsequent decimal dilutions in buffered peptone water were plated on adequate agar media for CFU determination. For each combination and each time of analysis, the mean and standard deviation of the three replicates were calculated for each sample. For a given time, values were considered to be significant between two series when a difference >0.5 Log_{10} CFU · g^{-1} was observed. To evaluate the evolution of target microorganisms (*S.* Typhimurium, *B. thermosphacta*, or *E. coli*) the Log_{10} difference between the end and beginning of storage ($CFU_{Tx} - CFU_{T0}$) was calculated, in order to obtain curves with an ordinate equal to zero at T0 and to facilitate the measurement of growth reduction between samples treated with *L. sakei* cocktails and untreated controls.