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Nutritionally enhanced fermented sausages as a vehicle for potential probiotic lactobacilli delivery

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ABSTRACT

The suitability of three potential probiotic lactobacilli strains (*Lactobacillus casei* CTC1677, *L. casei* CTC1678 and *Lactobacillus rhamnosus* CTC1679), previously isolated from infants' faeces and characterized, and three commercial probiotic strains (*Lactobacillus plantarum* 299v, *L. rhamnosus* GG and *L. casei* Shirota) was assessed during the manufacture of low-acid fermented sausages (*fuets*) with reduced Na^+ and fat content. The inoculated strains were successfully monitored by RAPD-PCR during the process. *L. rhamnosus* CTC1679 was the only strain able to grow and dominate (levels *ca.* 10^8 CFU/g) the endogenous lactic acid bacteria population in two independent trials, throughout the ripening process. Thus, *fuet* containing *L. rhamnosus* CTC1679 as a starter culture could be a suitable vehicle for putative probiotic bacteria delivery. All the final products recorded a satisfactory overall sensory quality without any noticeable off-flavour, and with the characteristic sensory properties of low-acid fermented sausages.

1. Introduction

Nowadays consumers expect food products to be safe and nutritious and demand healthy and natural foods (Jiménez-Colmenero, Reig, & Toldrà, 2006), including functional meat products with reduced levels of fat and sodium chloride content (Zhang, Xiao, Samaraweera, Joo Lee, & Ahn, 2010). The health benefits associated with probiotic food products are based on the presence of selected viable strains or microorganisms which, when administered in adequate amounts, improve the health of the host (FAO/WHO, 2001). The majority of microorganisms used for this purpose belong to the genus *Lactobacillus* and *Bifidobacterium*. Although dairy products are the most commonly used food vehicles for the delivery of probiotics, fermented sausages whose main microbiota consists of lactobacilli (Aymerich, Martín, Garriga, & Hugas, 2003) could be suitable products for the carriage of probiotic bacteria (Ammor & Mayo, 2007; Arihara, 2006). In addition, the sausage matrix offers protection for the survival of the probiotic lactobacilli through the gastrointestinal tract (Klingberg & Budde, 2006).

Many traditional fermented meat products with typical sensory characteristics are produced in Mediterranean countries. Among their traditional sausages, *fuet* is a type of small calibre low-acid fermented sausage (final pH > 5.3) from Catalonia (Northeast of Spain) made with pork meat, pork fat, and salt along with dextrose, nitrate, nitrite and pepper. The presence of inhibitors like salt, acidic pH and low water activity resulting from drying creates adverse conditions for the survival of probiotics (Khan et al., 2011). Thus, in selecting potentially probiotic bacteria as a starter culture one option would be to look for bacteria commonly associated with the meat environment that possess potential health promoting properties. Several investigations have been reported that deal with the use of bacterial strains isolated from sausages as probiotics in fermented meat products (Klingberg, Axelsson, Naterstad, Elsser, & Budde, 2005; Papamanoli, Tzanetakis, Litopoulou-Tzanetaki, & Kotzekidou, 2003; Pennacchia, Vaughan, & Villani, 2006; Pennacchia et al., 2004; Rebucci et al., 2007). To promote their survival in the host after ingestion another alternative could be the use of strains of human intestinal origin. Nevertheless these isolates are essentially required to survive fermentation and drying to be considered suitable for application in fermented sausages. In this way, strains of *Lactobacillus rhamnosus*, *Lactobacillus fermentum* and *Lactobacillus paracasei* of human intestinal origin have been shown to survive the acid fermented sausage manufacturing process and have been detected in high numbers in the final product (Erkkilä, Petajä, et al., 2001; Erkkilä, Suihko, Eerola, Petäjä, & Mattila-Sandholm, 2001; Pidcock, Heard, & Henriksson, 2002; Ruiz-Moyano, Martín, Benito, Hernández, et al., 2011). Not only bacterial survival is important but technological, sensory and safety aspects have to be assessed as well. In this respect, Erkkilä, Petajä, et al. (2001) and Erkkilä, Suihko, et al. (2001) showed that *L. rhamnosus* GG, LC-705 and E-97800 could be used during the manufacture of Northern European sausages without affecting the technological or sensory properties of the final product. Also it was observed in *Longaniza de Pascua*, a low-acid fermented sausage similar to *fuet*, and elaborated with the

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potentially probiotic strain *Lactobacillus casei* CECT475 (Sayas-Barberá, Viuda-Martos, Fernández-López, Pérez-Alvarez, & Sendra, 2012) that the inoculated strain reached counts *ca.* 10^9 CFU/g at the end of the process and although in the sensory analysis lower scores for flavour and colour intensity attributes were recorded, overall acceptability did not show significant differences from the control batch.

The aim of the present work was to assess the suitability of three potential probiotic lactobacilli strains (*L. casei/paracasei* CTC1677, *L. casei/paracasei* CTC1678 and *L. rhamnosus* CTC1679), previously isolated from infants' faeces and three commercial probiotic strains (*Lactobacillus plantarum* 299v, *L. rhamnosus* GG and *L. casei* Shirota) as starter cultures during the manufacture of low-acid fermented sausages (*fuets*) with reduced fat and Na^+ content and their effect on the sensory properties of the final product.

2. Materials and methods

2.1. Bacterial cultures

The lactobacilli strains used as probiotic starters were 3 potential probiotic strains selected for their safety, technological and probiotic features (*L. casei/paracasei* CTC1677 and CTC1678 and *L. rhamnosus* CTC1679). Briefly, these strains were chosen among 109 isolates for their ability to grow *in vitro* in the processing conditions of fermented sausages, further validated on model sausages, and for their functional and safety properties, including survival from gastrointestinal conditions (acidity, bile and pancreatin), no tyramine production, antibiotic susceptibility and aggregation capacity (Rubio, Jofré, Martín, Aymerich, & Garriga, in press) and 3 commercial probiotic strains (*L. plantarum* 299v, *L. rhamnosus* GG and *L. casei* Shirota) (Table 1). Each strain was grown overnight in de Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 37 °C, harvested by centrifugation at 9,600 ×*g* for 10 min at 6 °C, washed and re-suspended in saline solution (0.85% NaCl) and stored at −80 °C with 20% of glycerol until further use.

2.2. Fuets — manufacture and storage

Two independent trials (T1 andT2) were performed on two different days. The sausage mixture was prepared with lean pork and pork belly (75:25) and the following ingredients and additives (g/kg): NaCl, 15; KCl, 6.38; dextrose, 3; white pepper, 2; sodium ascorbate, 0.5; $NaNO_2$, 0.15; KNO_3, 0.15. To reduce the amount of Na^+, and according to previous results from Gou, Guerrero, Gelabert, and Arnau (1996), Gelabert, Gou, Guerrero, and Arnau (2003) and Guàrdia, Guerrero, Gelabert, Gou, and Arnau (2008), 25% of NaCl was substituted with KCl. To reduce the amount of fat, a lower proportion than usual of pork belly was used. Lean pork meat and pork belly were kept at −1 °C, minced in a meat cutter (Tecmaq, Barcelona, Spain) with a hole diameter of 6 mm and mixed with the other ingredients and additives in a mixing machine (model 35P, Tecnotrip S.A., Terrassa, Spain) for 2 min. The six lactobacilli cultures were prepared by diluting the stock at −80 °C in water (10 ml/kg) and were added to the meat batter to *ca.* 2×10^6 CFU/g to obtain the following batches: batch 1 (*L. casei/paracasei* CTC1677), batch 2 (*L. casei/paracasei* CTC1678), batch 3 (*L. rhamnosus* CTC1679), batch 4 (*L. plantarum* 299v), batch 5 (*L. rhamnosus* GG) and batch 6 (*L. casei* Shirota). The mixture was stuffed into natural pork casings. The *fuets* were dipped into a solution of *Penicillium candidum* spores (CHOOZIT™ Cheese Cultures, Danisco, France) and hung in a chamber where ripening took place at 12 °C and 75% relative humidity for 14 days (50% weight loss). After ripening, the *fuets* were packed in modified atmosphere (80% N_2/20% CO_2) in PET/PE pouches (Sacoliva SL., Spain) and stored at 4 °C for 74 days.

2.3. Physico-chemical parameters

At selected times the pH was determined directly in the product using a penetration electrode (Xerolit 52-32, Crison Instruments, S.A., Alella, Spain). Water activity (a_w) was measured with an Aqualab S3TE device (Decagon Devices, Inc. Pullman, Washington, USA). Chloride content was determined in the final products according to ISO 1841–2 (1996) using a potentiometric titrator 785 DMP Titrino (Metrohm AG, Herisau, Switzerland) and expressed as salt content. The total fat content of the final products was estimated by near infrared spectroscopy (Anderson, 2007) using a FoodScan™ Lab (Foss Analytical, Denmark).

2.4. Microbiological analysis

Microbiological analyses were performed in duplicate samples during the ripening process for LAB and Gram-positive Catalase-positive Cocci (GCC+) (days 0, 6 and 14) and after two months of storage (day 74) under MAP (80% N_2 and 20% CO_2) at 4 °C for LAB. For each sampling time, the casings were aseptically removed and 15 g of *fuet* was diluted 1/10 in dilution solution (0.1% Bacto Peptone (Difco Laboratories, Detroit, MI, USA) with 0.85% NaCl (Merck)) and homogenized in a Masticator Classic (IUL S.A., Barcelona, Spain) for 1 min. The homogenate was serially diluted and LAB counts were determined by plate counting in MRS (Merck) after 48 h at 37 °C in anaerobiosis (Anaero-Gen, Oxoid Ltd., Basingstoke, UK) and GCC+ were determined on Mannitol Salt Agar (MSA, Merck) after 48 h at 30 °C. Moreover, at the initial time (meat batter) and before the sensory analysis (final product) *Enterobacteriaceae* and *Escherichia coli* were determined by plate counting on Violet Red Bile Dextrose agar (VRBD, Merck) with a double layer at 37 °C for 24 h, ChromID Coli agar (bioMérieux S.A., Marcy l'Etoile, France) incubated at 37 °C for 48 h, respectively. *Listeria monocytogenes* and *Salmonella* were investigated in 25 g according to ISO 11290–1 (1996) and ISO 6579 (2002), respectively.

2.5. Identification of the inoculated lactobacilli strains

In order to monitor the inoculated lactobacilli strains, sixteen colonies of LAB per batch were randomly selected from the MRS agar plates at each sampling time and streaked in MRS agar. Isolated colonies were suspended in 200 μl of 6% chelating ion exchange resin Chelex®-100 (Bio-Rad, Hercules, CA, USA), heated at 100 °C for 10 min to release DNA from the cells and cooled on ice. The cell lysate was centrifuged at 14,000 ×*g* for 10 min and 50 μl of the supernatant containing the DNA was transferred to a new tube and stored at 4 °C until further use as a PCR template. The random primer KS (5′-tcgaggtcgacggtatcg) (Fulladosa et al., 2010) was used for RAPD analysis. Each 25-μl PCR reaction contained 2.5 μl of 10× PCR buffer, 1.5 mmol/l of $MgCl_2$, 0.2 mmol/l of each dNTP, 0.8 μmol/l of primer, 1 mg/ml of BSA, 1 U of TAQ polymerase (Invitrogen, Merelbeke, Belgium) and 2 μl of DNA. The amplification programme consisted of 2 min of initial denaturation at 95 °C and 40 cycles of denaturation at 95 °C for 30 s, annealing at 40 °C for 1 min and elongation at 72 °C for 1.5 min, followed by a final elongation at 72 °C for 7 min. Amplification products were analysed by the QIAxcel System (QIAGEN, Hilden, Germany) using a 12-channel QIAxcel DNA Screening Cartridge (separation performed using AM320 method). The size of the PCR products was scored with QIAxcel ScreenGel Software version 1.0.2.0 (QIAGEN).

2.6. Sensory evaluation

Six trained assessors (ASTM, 1981; ISO 8586–1, 1993; ISO 8586–2, 1994) carried out the sensory analysis on 5 mm-thick slices of *fuet*. The generation and selection of the descriptors had been carried out by open discussion in three previous sessions. The descriptors retained were: cured colour intensity (intensity of the cured colour of the lean