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Characterization of lactic acid bacteria isolated from infant faeces as potential probiotic starter cultures for fermented sausages

Raquel Rubio, Anna Jofré, Belén Martín, Teresa Aymerich, Margarita Garriga*

IRTA-Food Safety Programme, Finca Camps i Armet, E-17121 Monells, Girona, Spain

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ABSTRACT

A total of 109 lactic acid bacteria isolated from infant faeces were identified by partial 16S rRNA, *cpn60* and/or *pheS* sequencing. *Lactobacillus* was the most prevalent genus, representing 48% of the isolates followed by *Enterococcus* (38%). *Lactobacillus gasseri* (21%) and *Enterococcus faecalis* (38%) were the main species detected. A further selection of potential probiotic starter cultures for fermented sausages focused on *Lactobacillus* as the most technologically relevant genus in this type of product. Lactobacilli strains were evaluated for their ability to grow *in vitro* in the processing conditions of fermented sausages and for their functional and safety properties, including antagonistic activity against foodborne pathogens, survival from gastrointestinal tract conditions (acidity, bile and pancreatin), tyramine production, antibiotic susceptibility and aggregation capacity. The best strains according to the results obtained were *Lactobacillus casei/paracasei* CTC1677, *L. casei/paracasei* CTC1678, *Lactobacillus rhamnosus* CTC1679, *L. gasseri* CTC1700, *L. gasseri* CTC1704, *Lactobacillus fermentum* CTC1693. Those strains were further assayed as starter cultures in model sausages. *L. casei/paracasei* CTC1677, *L. casei/paracasei* CTC1678 and *L. rhamnosus* CTC1679 were able to lead the fermentation and dominate (levels *ca.* 10^8 CFU/g) the endogenous lactic acid bacteria, confirming their suitability as probiotic starter cultures.

1. Introduction

Probiotics have been reported to provide a wide variety of beneficial health effects and such effects have shown to be strain-dependent (De Vuyst et al., 2008; Sanders, 2008). The majority of the microorganisms used for probiotic purposes belong to the genus *Lactobacillus* and *Bifidobacterium*. Even though some species of these genera are "Generally Recognized As Safe" (GRAS) by the United States Food and Drug Administration (FDA) or have the "Qualified Presumption of Safety" (QPS) status by the European Food Safety Authority (EFSA), the working groups of the WHO/FAO recommend further studies to demonstrate that a particular probiotic strain is safe. In order to act as a probiotic in the gastrointestinal tract (GIT), a bacteria must be able to survive the acidic conditions of the stomach and resist the bile acids at the beginning of small intestine (Erkkilä and Petäjä, 2000). Lactic acid bacteria (LAB) are considered normal inhabitants of the GIT, thus intestinal strains should preferably be chosen to be used as probiotics (Saarela et al., 2000; Vizoso-Pinto et al., 2006). Other functional and safety properties used to characterise probiotics are the production of antimicrobial compounds, the adhesion to the intestinal mucosa (Ammor and Mayo, 2007; Martín et al., 2005b), the non production of biogenic amines, antibiotic susceptibility (Ammor and Mayo, 2007) and the ability to tolerate the presence of pancreatic enzymes (Rönkä et al., 2003).

Although dairy products are the most commonly used food vehicles for the delivery of probiotics, several investigations dealing with their use in different types of fermented sausages have been carried out (Erkkilä et al., 2001; Klingberg and Budde, 2006; Ruiz-Moyano et al., 2011a, 2011b). Additionally, other properties are demanded for probiotic cultures, such as desirable technological, sensory and safety features. Moreover, the probiotic culture should be well-adapted to the fermented sausage environment (*i.e.* presence of curing salts, acidity and low water activity) in order to compete with the endogenous microbiota and grow to levels that enable the display of health-promoting effects. Several investigations based on the screening of sausage isolates for probiotic features have been reported (Klingberg et al., 2005; Pennacchia et al., 2006; Rebucci et al., 2007; Ruiz-Moyano et al., 2008). For example, Rebucci et al. (2007) proposed strains of *Lactobacillus casei* and *Lactobacillus rhamnosus* isolated from salami as the best potential functional starter cultures in meat products. Pennacchia et al. (2006) confirmed the suitability of several strains of *Lactobacillus plantarum*, isolated from different types of Italian

* Corresponding author. Tel.: +34 972630052; fax: +34 972630373.
E-mail address: margarita.garriga@irta.cat (M. Garriga).

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fermented sausages, as probiotic cultures in meat products due to their *in vitro* adhesion capacity. Another alternative would be the use of strains of human intestinal origin for potential probiotic use in fermented sausages. These isolates are essentially required to survive fermentation and subsequent drying during sausage manufacturing. Accordingly, recent investigations have shown that strains of *L. rhamnosus*, *Lactobacillus fermentum* and *Lactobacillus paracasei* of human intestinal origin were able to survive the dry-sausage manufacturing process, being detected in high numbers in the final product (Erkkilä et al., 2001; Pidcock et al., 2002; Ruiz-Moyano et al., 2011b).

The main objective of this work was to perform a screening of LAB strains isolated from healthy infants' faeces for potential use in fermented sausages. Lactobacilli strains were assessed for their ability to grow *in vitro* in the processing conditions of fermented sausages and for their functional and safety properties including antagonistic activity against the foodborne pathogens *Listeria monocytogenes* and *Salmonella*. Strains were also tested for their survival to the GIT conditions (acidity, bile and pancreatin), biogenic amine production, antibiotic susceptibility and aggregation capacity. Additionally, the performance of six selected strains in a fermented sausage model was evaluated.

2. Materials and methods

2.1. Enumeration and isolation of LAB

The study included 43 faecal samples of healthy infants up to 6 months of age: 31 breast-fed, 4 formula-fed and 8 mixed-fed (breast and formula milk). The fresh faecal samples were collected at home by the parents and were processed directly from the nappies in the laboratory within 24 h of sample collection. For isolation of LAB, *ca.* 1 g of freshly collected faeces were resuspended in a saline solution (0.1% peptone and 0.85% NaCl), diluted, plated in MRS agar (pH 5.7, Merck, Darmstadt, Germany) and incubated under anaerobiosis at 37 °C for 48–72 h. One representative colony of each morphology (different in size, shape and/or colour from other colonies) was individually picked and streaked on MRS agar (Merck). Pure cultures were stored at −80 °C in 20% (v/v) glycerol.

2.2. Strain typing by RAPD-PCR

For each isolate, a loopful of cells grown on MRS agar was suspended in 200 μl of 6% Chelex®-100 (Bio-Rad, Hercules, CA, USA), heated at 100 °C for 10 min, cooled on ice and centrifuged at 14,000 g for 10 min. Fifty microlitres of the supernatants (containing the DNA) were used as PCR templates. RAPD-PCR analysis was performed with random primers KS, M13R2, R5 and CC1 (Table 1). Each 25-μl PCR reaction contained 2.5 μl of 10× PCR buffer, 3.5 mmol l^{-1} of $MgCl_2$, 0.2 mmol l^{-1} of each dNTP, 0.8 μmol l^{-1} of primer, 1 mg ml^{-1} of BSA, 1 U of TAQ polymerase (Invitrogen, Merelbeke, Belgium) and 2 μl of DNA.

The amplification program consisted of 2 min at 95 °C and 40 cycles of denaturation at 95 °C for 30 s (for M13R2 and KS) or for 1 min (for CC1 and R5), annealing for 1 min (Table 1) and elongation at 72 °C (1.5 min for M13R2 and KS and 2 min for CC1 and R5), followed by a final elongation at 72 °C for 5 min (primer M13R2) or 7 min (primers KS, CC1 and R5). GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA) was used.

PCR products were separated by the QIAxcel System (QIAGEN, Hilden, Germany) using a DNA Screening Cartridge and the AM320 separation method. RAPD profiles were normalised and analysed with InfoQuest™ FP software version 4.5 (Bio-Rad Laboratories, Hercules, CA, USA). Isolates from a single infant showing the same band pattern on RAPD analysis were considered to belong to the same genotype.

2.3. Identification of isolates

One or two isolates of each genotype were identified by sequencing V1–V3 regions of 16S rRNA. *Enterococcus*, *Lactobacillus*, and *Weissella* that could not be identified at species level, were identified by *cpn60* and/or *pheS* sequencing. *Streptococcus* species were identified by *sodA* sequencing. Each 50 μl PCR reaction contained 5 μl of 10× PCR buffer, 1.5 mmol l^{-1} of $MgCl_2$, 0.4 mmol l^{-1} of each dNTP, 0.5 μmol l^{-1} of each primer (Table 1), 2 U of TAQ polymerase (Invitrogen) and 5 μl of Chelex®-100 purified DNA. Amplicons were purified with the Qiacube device using the QIAquick PCR purification kit (QIAGEN) and 5 ng were amplified with the BigDye Terminator 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing was performed in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequences were analysed with on line BLAST-W2 and ClustalW from the European Bioinformatics Institute.

2.4. Growth assays

The growth of the lactobacilli strains was tested with Bioscreen C (Oy Growth Curves Ab Ltd, Helsinki, Finland) using the following broths: MRS-c (2%-glucose MRS (Merck)), MRS-Salt (2%-glucose MRS + 2.5% w/v NaCl), MRS-Nit (2%-glucose MRS + 150 ppm $NaNO_2$) and MRS-mix (0.7%-glucose MRS + 2.5% w/v NaCl + 150 ppm

Table 1
Primers and PCR conditions.

Primer	Sequence (5′–3′)	Target gene	Reference	T_A (°C)	Number of cycles
M13R2	GGAAACAGCTATGACCATGA	Random primer	Martín et al. (2005a)	38	40
KS	TCGAGGTCGACGGTATCG	Random primer	Fulladosa et al. (2010)	40	40
CC1	AGCAGCGTGG	Random primer	Cocconcelli et al. (1995)	33	40
R5	AACGCGCAAC	Random primer	Martín et al. (2005a)	29	40
BSF8	AGAGTTTGATCCTGGCTCAG	16S rRNA	Universal primers	50	40
BSF 343	TACGGGAGGCAGCAG				
BSF1541	AAGGAGGTGATCCAGCCGCA				
sodA1	CCITAYICITAYGAYGCIYTIGARCC	*sodA*	Poyart et al. (2000)	37	35
sodA2	ARRTARTAIGCRTGYTCCCAIACRTC				
H729	CGCCAGGGTTTTCCCAGTCACGACGAIIIIGCIGGIGAYGGIACIACIAC	*cpn60*	Brousseau et al. (2001)	50	40
H730	AGCGGATAACAATTTCACACAGGAYKIYKITCICCRAAICCIGGIGCYTT				
M13(-40)F	CGCCAGGGTTTTCCCAGTCACGAC			50	25
pheS-21-F	CAYCCNGCHCGYGAYATGC	*pheS*	Naser et al. (2005)	46	35
pheS-22-R	CCWARVCCRAARGCAAARCC				
pheS-23-R	GGRTGRACCATVCCNGCHCC			50	25