Features of Lactobacillus sakei isolated from Italian sausages: focus on strains from Ventricina del Vastese

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Abstract

In this study bacterial isolates from Ventricina del Vastese sausage, previously identified as Lactobacillus (L.) sakei, were characterised genotypically, physiologically and on the basis of some technologically relevant traits. A total of 70 L. sakei isolates from sausages manufactured with spontaneous fermentation in the same producing plant were taken into account. Six genotypic groups were distinguished on the basis of Rep-polymerase chain reaction with the GTG5 primer, some of which were found only in the sausages ripened at temperatures lower than 10°C for the first two months and lower than 16°C for the remaining three months, according to the traditional ripening process. Six strains were selected as representative of the genotypic profiles and further characterised. A high diversity in their fermentation profiles was observed, and different groups were separated on the basis of growth and acidifying capacity in meat extract. None of the strains produced histamine or tyramine in vitro. One strain was able to slightly inhibit Listeria (L.) monocytogenes and L. innocua and all six strains were able to slightly inhibit Enterobacteriaceae isolated from Ventricina del Vastese sausages in vitro. Results showed that most L. sakei strains can have a role in improving the safety of low acidifying fermented sausages, even though a limited acidifying capacity was observed in a meat-like substrate, and that L. sakei strains able to produce biogenic amines are unlikely to occur in spontaneously fermented meat products.

Introduction

Although initially isolated from rice wine (Kandler and Weiss, 1986), Lactobacillus (L.) sakei is the predominant Lactobacillus species in fermented meat and fish products and also in Southern European sausages (Montel, 1999). In fermented sausages, L. sakei is more competitive than other lactobacilli, showing a shorter lag phase, higher growth rate and higher cell numbers (Dossmann et al., 1996). L. sakei is psychrotrophic and can tolerate high salt concentrations; the capacity of some strains to grow at 4°C and in the presence of 6.5% NaCl, was reported (Ammon et al., 2005). The salt tolerance has been attributed to the ability of L. sakei to efficiently accumulate compatible solutes such as betaine and carnitine, and the cold tolerance was explained with the presence in the genome of L. sakei of more cold-stress genes than any other Lactobacillus spp. (Chaillou et al., 2005). L. sakei possesses a heme-dependent catalase (Ammon et al., 2005), which can prevent the green colour defect caused by H₂O₂ production by some lactic acid bacteria in meat products, and actively contributes to the hydrolysis of the sarcoplasmic proteins and to the subsequent decomposition of peptides into amino acids (Fadda et al., 1991). It has been demonstrated that L. sakei, for its rapid growth and acid production, greatly reduces the accumulation of biogenic amines (BAs) in fermented sausages by preventing the development of amine-producing bacteria (Bover-Cid et al., 2001). The occurrence of bacteriocinogenic strains of L. sakei inhibitory to Listeria (L.) monocytogenes has been known for long time (Shillinger et al., 1991), though the effect of non-bacteriocinogenic strains on this respect was little investigated. One study compared the antagonistic features of bacteriocinogenic and non-bacteriocinogenic L. sakei isolates toward bacterial contaminants when inoculated in fresh vacuum packed meat (Jones et al., 2009) but there are no data available on the antagonistic activity on non-bacteriocinogenic L. sakei strains in fermented meat products.

L. sakei has been isolated from human feces and it is therefore able to survive the passage through the human gastrointestinal tract (GIT) (Walter et al., 2001), the studies on its probiotic properties are underway (Park et al., 2008; Garriga et al., 2015). In a previous study on the identification of the pro-technological bacterial species isolated from Ventricina del Vastese, a traditional sausage of the Abruzzi and Molise regions made from pork meat cut into cubes of 2-5 cm sides, mixed with 20-25% lard, stuffed in natural casings to obtain sausages with 11-12 mm diameter and ripened for at least 150 days, it was found that all except one among 71 Lactobacillus spp. isolates belonged to the species Lactobacillus sakei, as ascertained by species-specific polymerase chain reaction (PCR) (Amadoro et al., 2013). The L. sakei isolates, obtained at different ripening times, came from sausages manufactured by a single producer and ripened in natural environmental conditions or in controlled conditions in plug-in showcase cabinets. The sausages ripened in the natural way, manufactured in December, had been exposed to temperatures lower than 10°C for the first 60 days and lower than 16°C for the remaining 90 days of ripening, while the sausages ripened in controlled conditions had been kept at 18°C in the first week of ripening and at 12°C later on. The predominance of the species L. sakei highlighted its importance for the traditional product considered and suggested to study the diversity of the isolates and the activities relevant for ripening and safety. Therefore, they were characterised genotypically by Rep-PCR and strains representing distinct genotypes were selected for the examination of physiological features. Moreover, safety aspects, such as the ability to inhibit Listeria spp. and Enterobacteriaceae isolated from Ventricina del Vastese and the absence of BAs production, were assessed in vitro.

Materials and Methods

Bacterial strains and culture methods

In this study 70 L. sakei isolates identified in a previous investigation were used. For routine sub-culturing the strains were propagated anaerobically in De Man Rogosa Sharp (MRS) broth (Bioflie Italiana Srl., Milan, Italy) at 30°C. Listeria monocytogenes ATCC 7644, L. monocytogenes 19111, L. innocua ATCC 33090 and 12 still unidentified Enterobacteriaceae isolates from Ventricina del Vastese sausage were propagated aerobically in Tryptone Soy Broth [TSB; Oxoid Spa., Rodano (MI), Italy] at
30 and 37°C, respectively. Fermentation profiles were obtained using the API 50 CHL system [BioMerieux Italia Spa., Bagno a Ripoli (FI), Italy], after incubation for 48 h at 30°C in anaerobiosis.

The strains of L. sakei were inoculated in a culture broth consisting of 10 g/L of Lab Lemco powder (Oxoid) to assess the growth capacity in different conditions in a substrate similar to meat. The cultures were then incubated at 28°C for 48 h.

The acidifying ability was determined by measuring the pH of 10 g/L Lab Lemco suspensions inoculated with 1% (v/v) of fresh culture at 0, 3, 4, 6, 12, 24, 48 and 72 h. Growth was followed by measuring the OD600 with a UV/Vis Spectrometer Jasco V-530 [Jasco Europe Srl., Cremella (LC), Italy].

The capacity to grow at 5 and 15°C and in presence of salt at concentrations 0, 5, 7.5 and 10% w/v was tested during an incubation period of 14 days. Growth was considered to be positive when an OD600 of at least 0.150 was reached.

The type of fermentative metabolism was assayed in homofermentative-heterofermentative differential (HHD) broth (Biolife Italiana Srl.).

The ability to produce histamine and tyramine was assayed as described by Joosten and Northolt (1989). The antagonistic activity was tested by the agar spot deferred method on MRS modified according to Schillinger and Lücke (1989), i.e. with ten fold less glucose (final concentration 2 g/L), as follows: 5 µL of L. sakei fresh culture were placed on the surface of modified MRS in a Petri plate and let to develop overnight aerobically at 30°C. Then 5 mL of tryptic soy agar (TSA), containing 5 g/L agar, inoculated with 50 µL fresh culture of indicator strain, Listeria spp. or Enterobacteriaceae isolates, were poured on the modified MRS with the L. sakei spots. Incubation was carried out for 48 h at 30°C and at 37°C for Listeria spp. and Enterobacteriaceae, respectively.

Molecular techniques
Genomic DNA was extracted from 1 ml of fresh bacterial culture by the Genomic DNA Extraction kit, according to manufacturer’s protocol (RBC Bioscience, New Taipei City, Taiwan). For genotyping of the 70 L. sakei isolates, the Rep-PCR with the primer GTG5 and agarose gel separation were applied as described by Versalovic et al. (1994). Identification to species level was confirmed by the PCR test reported by Berthier and Ehrlich (1999) and by sequencing of the 16S rRNA gene. Amplification of a region of the 16S rRNA gene of c.a. 1500 bp was carried out according to Bringel et al. (2005) and sequencing was done on the PCR fragments purified with the HiYield Gel/PCR Fragment Extraction Kit (RBC Bioscience) by Eurofins Genomics (Ebersberg, Germany) with the same oligonucleotides used for the amplification. The species identification was obtained by BLAST alignment with the public database.

Results
Seventy L. sakei isolates (Amadoro et al., 2013) were typed by Rep-PCR and six genotypes could be distinguished, so that six strains, each representative of one genotype, were retained for further characterisation (Figure 1). Noticeably, most of the strains with similar profiles, namely L. sakei LS1, LS2, and LS3 were isolated from sausages ripened in controlled conditions, while L. sakei LS4 and...
Table 1. Physiological characteristics of *Lactobacillus sakei* strains isolated from *Ventricina del Vastese* sausages.

| Physiological characteristics | 1   | 2   | 3   | 4   | 5   | 6   |
|-----------------------------|-----|-----|-----|-----|-----|-----|
| Sorbitol                    | -   | -   | -   | -   | -   | +   |
| Methyl-αD-mannopyranoside   | -   | -   | -   | -   | -   | +   |
| Methyl-αD-glucopyranoside   | -   | -   | +   | -   | -   | -   |
| Amylose                      | -   | -   | -   | -   | -   | +   |
| Arbutin                      | +   | +   | +   | -   | +   | -   |
| Salicin                      | +   | +   | +   | -   | +   | -   |
| D-Cellobiose                 | +   | +   | +   | -   | +   | -   |
| D-Maltose                    | -   | -   | -   | -   | -   | +   |
| D-Lactose                    | -   | -   | -   | -   | -   | +   |
| D-Melitose                   | -   | -   | -   | -   | -   | +   |
| D-Raffinose                  | ±   | ±   | ±   | ±   | ±   | ±   |
| Gentiose                     | ±   | ±   | ±   | ±   | ±   | ±   |
| Potassium gluconate          | ±   | ±   | ±   | ±   | ±   | ±   |
| D-Turanose                   | ±   | ±   | ±   | ±   | ±   | ±   |
| D-Arabinol                   | ±   | ±   | ±   | ±   | ±   | ±   |
| NaCl 10%, 5°C                | -   | -   | -   | -   | -   | -   |
| NaCl 5%, 5°C                 | -   | -   | -   | -   | -   | -   |
| NaCl 10%, 5°C                | -   | -   | -   | -   | -   | -   |

±, uncertain reaction. Only traits found to be variable are reported.

**Discussion**

The genetic heterogeneity of *L. sakei* has been reported for strains isolated from different sources, i.e., meat, fish and plant material (Chaillou et al., 2009). However, in this study, the occurrence of different biotypes was demonstrated even in the same product from a single producing plant. In addition, it was shown that different environmental conditions can drive the selection of particular strains. The results are in agreement with the low levels of genetic relatedness among *L. sakei* strains found in other studies that proposed two subspecies, *L. sakei* subsp. *sakei* and *L. sakei* subsp. *carnosus*, and, more recently, three main evolutionarily divergent lineages (Klein et al., 1996; Torriani et al., 1996; Chaillou et al., 2009, 2013). However, the distinction at the subspecies level was not investigated in this study, since the molecular techniques applied were different from those proposed to attribute the *L. sakei* strains to the two subspecies and biochemical tests cannot be used to this purpose (Klein et al., 1996).

The strains examined exhibited also physiological variability, in accordance with that observed in other studies reporting phenotypic differences that rendered difficult the classification of *L. sakei* strains on the basis of fermentation profiles (Klein et al., 1996; Torriani et al., 1996).

Strain *L. sakei* LS6 had the widest fermentation spectrum and was able to ferment substrates not previously reported for *L. sakei* strains (Nyquist et al., 2011). Moreover, the ability of *L. sakei* LS5 and LS6 to ferment lactose, that was previously observed for strains isolated from Spanish sausages (Nyquist et al., 2011), is interesting for application in sausages where this sugar is added. A particular finding is the heterofermentative-like behaviour of *L. sakei* LS5 in HHD medium, considered that such phenotype was not observed before (Ammor et al., 2005).

Strain *L. sakei* LS6 is the most versatile among the biotypes characterised in this study, and also the one that permitted a better and
long lasting acidification in meat extract (Figure 2). However it exhibited a low tolerance to NaCl (Table 1). This may be the reason why it was retrieved only from sausages ripened at low temperature according to the traditional process, where slower drying may have favoured its persistence.

The strains studied exhibited some capacity to inhibit the growth of Enterobacteriaceae and strain LS5 also slightly inhibited Listeria spp. Therefore a general capacity of L. sakei to contrast the growth of Enterobacteriaceae in fermented meat products can be hypothesised and requires confirmation by testing different species and by examining their inhibition during sausage ripening. On the other hand, evidence that L. sakei strains, even if non bacteriocinogenic, are capable to retard spoilage and to reduce the number of bacterial pathogens by developing dominant populations is presently available for vacuum packaged fresh meat (Jones et al., 2009). An explanation for the inhibitory effects exerted by non bacteriocinogenic strains might be the competition for nutrients and better colonising ability of meat, given the specialisation of L. sakei for this ecological niche (Nyquist et al., 2011). However, a low level of bacteriocin production cannot be ruled out for the strains examined in this study and should be investigated by PCR based assays for the presence of bacteriocin encoding genes and, eventually, for their expression during sausage ripening. Indeed, on the basis of what reported by Todorov et al. (2013), the low inhibition effect observed in this study might be due to a low bacteriocin production determined by the low concentration of glucose in the test medium.

In particular, the ability to produce bacteriocins should be investigated for L. sakei LS5, which exhibited a slight anti-listerial effect in vitro. Indeed, recent findings confirmed that the anti-listerial potential of bacteriocinogenic L. sakei represents an efficient tool to contrast the development of L. monocytogenes in food even at low temperatures (Martínez et al., 2015).

Evidences obtained in this study indicated that for an appropriate evaluation of the role of L. sakei on Ventricina del Vastese microbiological safety, challenge tests with different bacterial contaminants, co-inoculated with single strain or combined L. sakei cultures, should be carried out.

Finally, the inability to form BAs is in line with the fact that this physiological trait was never reported to occur in L. sakei strains. The capacity of autochthonous L. sakei strains to become dominant in the microbial population associated to Ventricina del Vastese sausage suggested that they can reduce the risk of accumulation of these hazardous compounds also in this product by outcompeting BA forming bacterial species.

Conclusions

This study represents a contribution for knowledge on the genotypic, phenotypic and technological characteristics of L. sakei bio-types occurring in Italian sausages. It emerged that the L. sakei population of a traditional sausage production, for the same manufacturer and for a single production cycle, is genotypically and phenotypically heterogeneous and that some strains could be isolated only in the product which was subjected to the traditional ripening process at low temperature. The limited growth and pH lowering capacity shown by most strains in pure cultures and in meat extract alone suggests that these bacterial species need nutritional sources that are made available by other components of the sausage microbiota for optimal development. However, some strains can be selected, such as L. sakei LS6 that are endowed with a good acidification capacity.

All strains were able to slightly inhibit Enterobacteriaceae isolated from the same product by mechanisms different from acidification. This suggested the possibility that autochthonous L. sakei strains play a role in the decline of such microbial groups and prevent the formation of undesired metabolites. However, most strains did not inhibit L. monocytogenes, indicating that only some L. sakei strains can have an effect in limiting this pathogen in the product.

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