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 GTG_5 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 acidity 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 (Ammor *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 (Ammor *et al*., 2005), which can prevent the green colour defect caused by H_2O_2 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*., 1999). 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 packaged 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*.

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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 (Biolife 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 OD_{600} 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 OD_{600} 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 GTG_5 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 r*RNA* 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

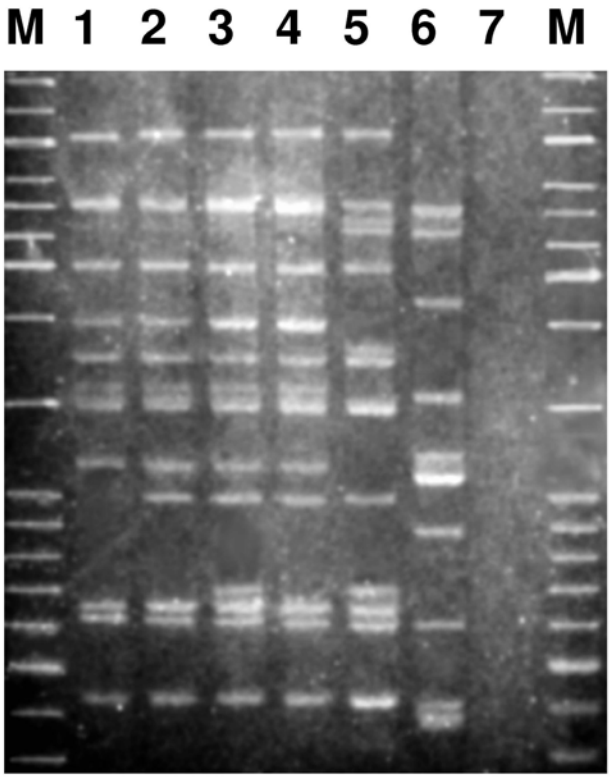

Figure 1. Electrophoretic profiles corresponding to the six strains representative of the six *Lactobacillus sakei* genotypes (lane 1, strain LS1; lane 2, strain LS2; lane 3, strain LS3; lane 4, strain LS4; lane 5, strain LS5; lane 6, strain LS6) identified in *Ventricina del Vastese* sausages manufactured in the same producing plant. Lane 7, negative control; M, mixture of GeneRuler 1 kb Plus and 100 bp DNA Ladders [Carlo Erba Reagents, Cornaredo (MI), Italy].

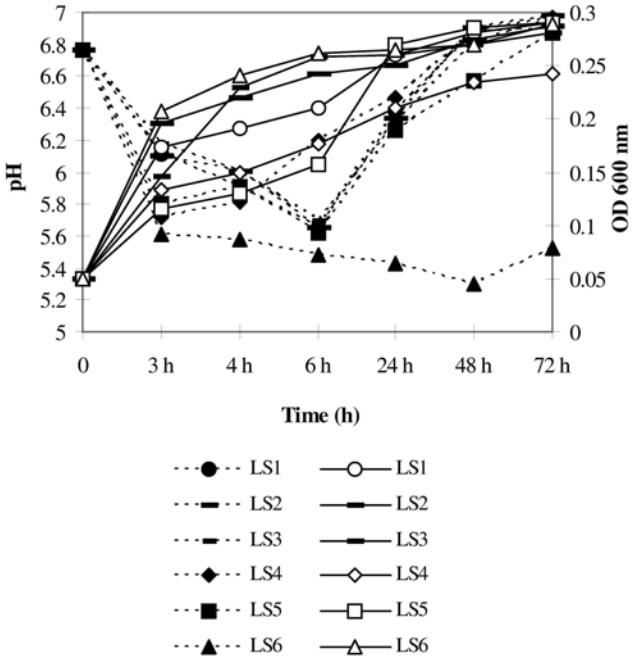

Figure 2. Growth and acidification of *Lactobacillus sakei* strains in 1% (w/v) meat extract suspension. Open symbols correspond to growth levels and solid symbols to pH values. Data are the average of two measurements.

Table 1. Physiological characteristics of *Lactobacillus sakei* strains isolated from *Ventricina del Vastese* sausages.

Physiological characteristics	Strain					
	1	2	3	4	5	6
Sorbitol	-	-	-	-	-	+
Methyl-αD-mannopyranoside	-	-	-	-	-	+
Methyl-αD-glucopyranoside	-	-	+	-	-	-
Amylose	-	-	-	-	-	+
Arbutin	-	-	-	-	-	+
Salicin	-	-	-	-	+	+
D-Cellobiose	+	+	+	+	-	+
D-Maltose	-	-	-	-	-	+
D-Lactose	-	-	-	-	+	+
D-Melezitose	-	-	-	-	-	+
D-Raffinose	-	-	-	-	-	+
Gentiobiose	±	±	+	±	-	+
Potassium gluconate	±	±	±	±	±	+
D-Turanose	-	-	-	-	-	+
D-Arabitol	-	-	-	-	-	±
NaCl 10%, 5°C	+	+	+	-	+	-
NaCl 5%, 15°C	+	+	+	-	+	+
NaCl 10%, 15°C	+	+	+	-	+	-

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

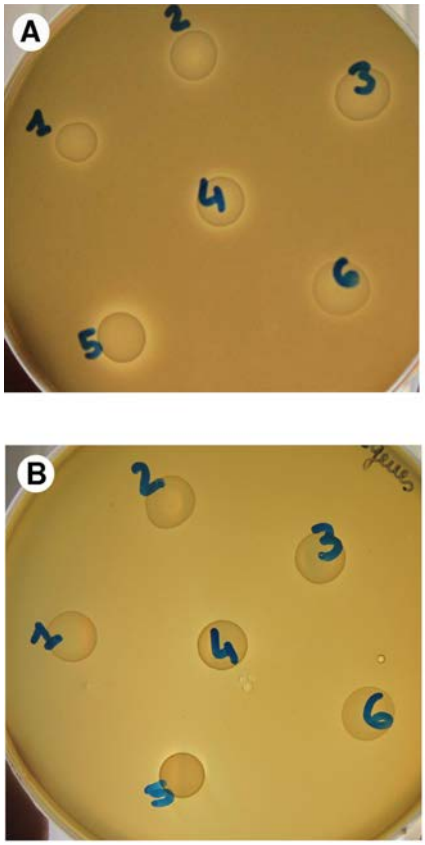

Figure 3. Antagonistic effect of the six *Lactobacillus sakei* strains *in vitro* against an *Enterobacteriaceae* isolate (A) and *Listeria monocytogenes* ATCC 19111 (B).

the most genetically dissimilar, *L. sakei* LS5 and LS6, that represented a minority of the isolates, 5 and 3, respectively, were isolated from sausages ripened in the traditional way (Amadoro *et al*., 2013).

The strains exhibited also distinct fermentation profiles showing different carbohydrates utilisation profiles (Table 1). All fermented D-Ribose, D-Galactose, D-Glucose, D-fructose, D-Mannose, N-Acetylglucosamine, Amygdalin, D-Melibiose, Sucrose, D-Trehalose and hydrolysed Esculin ferric citrate. *L. sakei* LS6 exhibited the widest substrate utilisation capacity. All strains gave a homofermentative reaction when grown in HHD medium except *L. sakei* LS5. The ability to grow at temperature as low as 5°C in meat extract suspension was common to all strains, however *L. sakei* LS4 and LS6 were inhibited by salt in this medium, even at the lowest concentration assayed of 5% (w/v). *L. sakei* LS4 was inhibited by 5% (w/v) NaCl also at 15°C, while strain LS6 was not (data not shown). The six strains showed different growth behaviors in meat extract, and their growth capacity was limited in this medium. All strains except LS6 acidified the medium with a similar trend, comprising a rapid drop of about 1 pH unit in 3 h and a new increase to the initial levels starting after 6 h. Only for strain *L. sakei* LS6, pH reached lower values and remained rather stable until 72 h (Figure 2). None of the strains produced histamine or tyramine *in vitro*.

The *L. sakei* strains were tested for inhibiting capacity against 12 *Enterobacteriaceae* isolates previously obtained from *Ventricina del Vastese* sausage at 10 days of ripening (unpublished), and against *L. monocytogenes* and *L. innocua* strains. None of the strains showed a strong inhibiting capacity. Nevertheless, a small inhibition zone was visible around all the *L. sakei* spots in the plates inoculated with all the *Enterobacteriaceae* isolates tested as in the example shown in Figure 3A. Very slight inhibition towards *L. monocytogenes* and *L. innocua* was noticed only for *L. sakei* LS5 (Figure 3B).

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 (Martinez *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* biotypes 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 authochtonous *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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