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Characterization and selection of *Lactobacillus sakei* strains isolated from traditional dry sausage for their potential use as starter cultures

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Abstract

A total of 36 *Lactobacillus sakei* strains isolated from traditional dry sausage, produced without the use of starter cultures, were characterized in respect to their technological properties, i.e. growth and acidification kinetics, ability to grow at different temperatures, pH values or salt concentrations, gas production from glucose, heme-dependent catalase activity, inhibitory activity against pathogens and proteolytic and lipolytic activities.

Growth rates were comprised between 0.12 and 0.55 gen/h; while acidification rates varied from 0.01 and 0.07 pH unit/h. All isolates were able to grow at pH 4.2–9.6 and at 15 °C on acetate agar supplemented with 4% NaCl, but were not able to grow at 0 and 45 °C and with 10% NaCl in the same pH conditions. However, 97% of the isolates were able to grow at 4 °C, but only 11%, and 55% were able to grow at pH 3.9 and with 6.5% NaCl, respectively. All isolates were homofermentative from glucose. Heme-dependant catalase activity was found for 97% of the isolates and 3% displayed antibacterial activity against *Listeria innocua*. Most isolates showed leucine and valine arylamidase and only one exhibited a lipase (C14) activity.

Two strains were selected using a multi-variate analysis. Both of them showed a panel of properties highly desirable in sausage fermentation. We propose the use of these strains, in combination with coagulase-negative staphylococci as starter cultures in the studied small-scale facility in order to improve and standardize sausage safety and quality while preserving their typicality.

Keywords: Lactic acid bacteria; *Lactobacillus sakei*; Traditional dry sausage; Meat small-scale facility; Starter culture; Typicality; Selection; Characterization

1. Introduction

Traditional fermented food products of meat origin are produced by numerous small-scale and family sized processing units in Europe. Their fermentation relies on natural contamination by environmental flora. This contamination occurs during animal slaughtering and increases during manufacturing. Fermentation is not controlled. Each small-scale facility has a specific house flora composed of useful micro-organisms for the fermentation and flavor of sausages, as well as of spoilage and pathogenic flora.

The diversity of natural resources, traditions, competition, and income levels of the agricultural sector are emphasized by the European Union in Agenda 2000 (Commission of European Communities, 1999). However, both consumer demand and the framework of the application of regulations, laws and regulatory bodies impose limits on practice, ensuring that safe products are delivered. Food safety is a top priority for the European Community, as indicated in the White Paper on Food Safety (Commission of European

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Communities, 2000), and it is regulated by Commission of European Communities directive 93/43/CEE (Council of the European Communities, 1993). Therefore, traditional fermented food products producers have the obligation to deliver safe products. Likewise, most of decontaminating solutions used for sanitization of processing equipments and environmental surfaces are harsh, killing most of the micro-organisms including those that might have a positive function such as fermentation or inhibition of undesired bacteria. For traditional fermented products, the destruction of the typical house flora may thus lead to manufacturing problems and significant losses of flavor and typicality of these products (Ammor et al., 2004a).

Improving traditional dry sausage safety and typicality may be achieved by the conjunction of two approaches. First, by the introduction of selective decontaminating procedures targeted towards spoilage and pathogenic bacteria, but preserving technological flora (Ammor et al., 2004a), second, by the addition of starter cultures specially selected from the small-scale facility house flora in order to control the fermentation process and thus to improve products safety and technological quality, while preserving their typicality.

According to the definition of Hammes (1996), meat starter cultures are 'preparations which contain living or resting micro-organisms that develop the desired metabolic activity in the meat'. They are, by definition, used to change the sensory properties of the food. In meat fermentations, lactic acid bacteria (LAB) have generally three different purposes, namely (i) to improve safety by inactivating pathogens, (ii) to improve the product stability and shelf life by inhibiting undesirable changes brought about by spoilage micro-organisms or abiotic reactions, and (iii) to provide diversity by the modification of raw material to obtain new sensory properties (Lücke, 2000). For the last purpose, other micro-organisms than LAB, namely, catalase-positive cocci (*Staphylococcus*, *Kocuria*), yeasts (*Debaromyces*) and moulds (*Penicillium*) are involved in the production and stability of the desired sensory properties (Lücke, 2000).

LAB originating from fermented meats are particularly well adapted to the ecology of meat fermentation (Hugas and Monfort, 1997). Thus, meat starter cultures should be selected from these meat LAB. The first stage in the starter culture designing process is to characterize LAB isolated from the given meat products, in order to select the best strains. We recently investigated the microbial flora of a small-scale sausage production unit, and showed that among LAB, *Lactobacillus sakei* was the predominant species present in meat (Ammor et al., 2004b). Therefore, the aim of this study is to characterize and select *L. sakei* strains showing the best technological properties, amongst those isolated from a traditional dry sausage production unit without the use of commercial starter cultures.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The 36 *L. sakei* strains used for the study were isolated from a small-scale facility producing traditional dry sausage without addition of starter cultures. These isolates were collected by Chevallier et al. (2001) and genotypically identified by Ammor et al. (2004b) as belonging to the *L. sakei* species. Among these isolates 16 (E1L-20–35) and 19 (E1L-1–16) were originating from sausage after 1 (S1W) and 9 (S9W) weeks, respectively, of processing, and one (E1L-36) had been isolated from knife (K) (Chevallier et al., 2001). Bacteria were kept frozen at −20 °C in MRS broth (Biokar Diagnostics, Beauvais, France) supplemented with 10% glycerol.

Listeria innocua ATCC 33090 and *Staphylococcus aureus* E1S-5, a strain isolated from the same small-scale facility, were grown in BHI medium and used as indicator micro-organisms to test antagonistic activity of *L. sakei* strains. An agar well diffusion assay (Schillinger and Lücke, 1989) was used for this purpose.

2.2. Growth and acidification kinetics

Growth and acidification kinetics of *L. sakei* strains were tested in SB medium designed for simulating some technological conditions of sausage manufacturing. The SB medium contained 10% meat extract (Biokar Diagnostics), 2% D-glucose (Sigma-Aldrich Chemie, Steinheim, Germany), 2.5% NaCl (Merck-eurolab, Briave le Canal, France) and 1% bactotryptone (BD, Le Pont de Claix, France). The pH of the medium was adjusted to 6.5.

Each isolate was propagated overnight in MRS broth at 30 °C. Cells were first grown 6 h in 15 ml MRS broth at 30 °C and then inoculated (1% inoculum) to 15 ml MRS broth and incubated at 30 °C. The optical density (OD) of each culture was followed at 600 nm with a Cary 100 Bio UV–Visible spectrophotometer (Varian, Australia). When bacterial suspensions reached an OD_{600} value of 0.55 ± 0.02, a defined volume was used to inoculate 75 ml of SB broth to obtain an $OD_{600} = 0.05$. Cells were incubated at 15 °C and the temperature recorded in the studied small-scale facility. OD_{600} and pH values were recorded after 0, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 48 h of incubation. pH values were recorded using a microelectrode (SenTix, WTW, Germany). Experiments were performed twice and results are expressed as the mean value ± standard deviation.

2.3. Characterization of physiological properties

Enzymic activities of *L. sakei* strains were assayed using the API ZYM galleries (BioMérieux, Montalieu-Vercieu, France) as described by the manufacturer.

Growth at different temperatures was observed after 3 days of incubation at 15 and 45 °C, or 5 days at 0 and 4 °C on MRS agar plates. Growth at different pH values was observed after 3 days of incubation at 30 °C on MRS agar plates adjusted with HCl (1N) or NaOH (1N) to pH 3.9, 4.2, 5.2, 9.0, and 9.6. Growth on acetate agar (AA) was observed after 3 days of incubation at 30 °C. Growth at different salt concentrations was observed after 3 days of incubation at 30 °C on MRS agar plates added with 4%, 6.5%, or 10% NaCl.

Gas production from glucose was determined in ADAC medium as described by Sperber and Swan (1976).

Except enzymic assay, which was performed once, experiments were performed in triplicate and the results are expressed as the mean value of the three experiments.

2.4. Detection of the presence of the katA gene

L. sakei strains were screened by PCR for the presence of the *katA* gene, encoding heme-dependent catalase. The following specific primers were used for this purpose: 702-F (AATTGCCTTCTTCCGTGTA, position 551–536) and 310-R (AGTTGCGCACAATTATTTTC, position 127–139). These primers were designed based on the published sequence of the catalase gene katA of Lactobacillus sakei LTH 677 (Knauf et al., 1992). Chromosomal DNA used for PCR amplification was prepared by the method of Anderson and McKay (1983). PCR reactions were performed in PTC-100 thermal cycler (MJ Research, Massachusetts, USA) in a total volume of 25 µl containing 1 × PCR buffer + NH_2-SO_4 (MBI Fermentas, Mundolsheim, France), 1.5 mmol/l $MgCl_2$ (MBI Fermentas), 0.2 $mmol^{-1}$ of each dNTP (MBI Fermentas), 0.8 µmol/l of each primer (Sigma-Genosys, Cambridge, UK), 2U of *Taq* DNA polymerase (MBI Fermentas) and 1 µg/ml of DNA. The amplification consisted in an initial denaturation step at 94 °C for 5 min, followed by 30 cycles (denaturation at 94 °C, 1 min; primer annealing at 56 °C, 1 min; primer extension at 72 °C, 1 min), a final extension step at 72 °C, and cooling to 4 °C for 7 min. Negative controls without DNA template were included in parallel. Four microliters of the PCR products were submitted to electrophoresis at 100 V/cm for 1 h on a 1.5% agarose gel (Qbiogene, Illkirch, France) in 0.5 × Tris-acetate-EDTA buffer stained with 1.3 mmol/l of ethidium bromide (Qbiogene). A 100-bp DNA ladder (Jules™, Qbiogene) was used as molecular size marker.

2.5. Statistical analysis

A principal component analysis (PCA) was applied to growth and acidification data obtained for all *L. sakei* isolates. Alternatively, an Agglomerative Hierarchical Clustering (AHC) was used to select the *L. sakei* isolates that can play the role of meat starter cultures. The computer software used was XLSTAT (Addinsoft, Paris, France).

3. Results and discussion

3.1. Growth and acidification kinetics

Table 1 summarizes growth and acidification properties of each isolate. Growth lag phase was comprised between 14 and 22 h; while acidification lag phase was about 22 h for most of the isolates. Growth rates were comprised between 0.12 and 0.55 generation per hour; while acidification rates varied from 0.01 to 0.07 pH unit per hour. The OD_{600} reached after 48 h extends from 0.25 to 0.78; while the pH values reached 4.84–5.57. A correlation was observed between the final OD reached after 48 h incubation and pH value.

A normalized PCA was carried out on growth and acidification parameters data. Results are shown in Fig. 1. The PC 1 which took into account 62.4% of the total variance discriminates between strains isolated from the final product (S9W), and those from both S1W (sausage after 1 week of fermentation process) and from the knife. Strains belonging to the S9W group were characterized by a longer growth lag phase, but exhibited the fastest growth and acidification rates. This observation suggests a possible succession of several types of *L. sakei* populations. A first population would set-up the fermentation process while a second would ensure and monitor the process.

3.2. Physiological characterization

The ability of the 36 isolates to grow at different salt concentrations (4%, 6.5%, and 10%), temperatures (0, 4, 15, and 45 °C), pH values (3.9, 4.2, 5.2, 9, and 9.6), and on AA medium, as well as gas production from glucose are reported in Table 2. All isolates were able to grow on acetate medium, at 15 °C, at pH 4.2, 5.2, 9 and 9.6, and in the presence of 4% NaCl. In contrast, none could grow at 0, 45 °C and with 10% NaCl. Gas production from glucose was not detected with any isolate. Differences were observed in only three conditions: growth at 4 °C, with 6.5% NaCl, or at pH 3.9. Indeed, of the 36 isolates only one did not show any detectable growth at 4 °C, 16 did not grow with 6.5% NaCl, and only four were able to grow at pH 3.9. Similar results have been reported by Papamanoli et al. (2003) who showed that among 49 *L. sakei* strains isolated from traditional dry sausages, 88% were able to grow at 4 °C, 100% at 15 °C, 35% at 45 °C, 0% at pH 4.0 and 80% with 6.5% NaCl.

Table 1
Growth and acidification kinetics parameters of *L. sakei* isolates

Strain name	Origin	G_L (h)	G_K (gen/h)	OD 48 h	A_L (h)	A_K (pH unit/h)	pH 48 h
E1L-1	S9W	22±0	0.44±0.01	0.63±0.06	22±0	0.03±0.01	5.33±0.02
E1L-2		20±0	0.26±0.01	0.33±0.06	22±0	0.06±0.00	4.86±0.02
E1L-3		22±0	0.25±0.09	0.54±0.03	22±0	0.03±0.01	5.12±0.00
E1L-4		22±0	0.38±0.05	0.55±0.10	22±0	0.05±0.01	4.90±0.03
E1L-5		22±0	0.32±0.04	0.33±0.03	22±0	0.04±0.00	4.92±0.00
E1L-6		21±1	0.30±0.04	0.42±0.06	22±0	0.03±0.01	5.17±0.01
E1L-7		21±1	0.43±0.02	0.39±0.01	22±0	0.04±0.00	5.00±0.04
E1L-8		22±0	0.55±0.09	0.50±0.07	22±0	0.03±0.00	5.18±0.02
E1L-9		22±0	0.41±0.02	0.52±0.02	22±0	0.05±0.00	5.09±0.09
E1L-10		22±0	0.27±0.07	0.53±0.00	22±0	0.04±0.00	5.34±0.02
E1L-11		22±0	0.33±0.02	0.55±0.02	22±0	0.04±0.00	5.18±0.06
E1L-12		22±0	0.34±0.04	0.34±0.05	22±0	0.05±0.00	4.88±0.01
E1L-13		22±0	0.23±0.01	0.42±0.02	22±0	0.05±0.00	4.84±0.04
E1L-14		21±1	0.19±0.06	0.55±0.02	22±0	0.05±0.00	4.92±0.01
E1L-15		22±0	0.30±0.03	0.44±0.07	22±0	0.02±0.01	4.97±0.05
E1L-16		22±0	0.17±0.05	0.29±0.01	22±0	0.05±0.00	4.89±0.03
E1L-17		20±0	0.22±0.02	0.45±0.01	22±0	0.04±0.00	5.18±0.00
E1L-18		22±0	0.31±0.09	0.30±0.01	22±0	0.03±0.01	5.18±0.01
E1L-19		22±0	0.31±0.00	0.25±0.06	22±0	0.07±0.01	4.90±0.03
E1L-20	S1W	19±1	0.14±0.02	0.78±0.02	20±0	0.02±0.00	5.36±0.01
E1L-21		15±1	0.17±0.00	0.73±0.00	22±0	0.03±0.00	5.40±0.01
E1L-22		14±0	0.15±0.00	0.78±0.01	22±0	0.03±0.00	5.37±0.02
E1L-23		20±0	0.12±0.00	0.71±0.00	19±1	0.02±0.00	5.55±0.04
E1L-24		17±1	0.15±0.00	0.78±0.00	22±0	0.04±0.00	5.25±0.01
E1L-25		16±0	0.15±0.01	0.73±0.03	22±0	0.03±0.00	5.40±0.02
E1L-26		18±0	0.14±0.00	0.72±0.02	21±1	0.04±0.01	5.33±0.02
E1L-27		16±0	0.16±0.01	0.74±0.01	20±0	0.03±0.00	5.36±0.03
E1L-28		18±0	0.16±0.01	0.54±0.00	22±0	0.02±0.00	5.72±0.00
E1L-29		16±0	0.14±0.01	0.77±0.02	22±0	0.04±0.00	5.35±0.00
E1L-30		17±1	0.12±0.01	0.75±0.01	22±0	0.04±0.00	5.37±0.01
E1L-31		17±1	0.17±0.01	0.75±0.00	22±0	0.03±0.00	5.30±0.00
E1L-32		18±0	0.15±0.02	0.72±0.03	20±0	0.02±0.00	5.30±0.01
E1L-33		15±1	0.17±0.02	0.72±0.01	18±0	0.03±0.00	5.57±0.01
E1L-34		18±0	0.20±0.01	0.63±0.03	20±0	0.03±0.00	5.31±0.00
E1L-35		19±1	0.16±0.00	0.74±0.03	22±0	0.01±0.00	5.36±0.00
E1L-36	K	18±0	0.16±0.01	0.77±0.00	22±0	0.03±0.00	5.07±0.02

G_L: growth lag phase; G_K: growth rate; OD 48 h: optical density reached after 48 h; A_L: acidification lag phase; A_K: acidification rate; pH 48 h: pH value reached after 48 h; S9W: sausage after 9 weeks of processing; S1W: sausage after 1 week of processing; and K: Knife.
Values are expressed as the mean±standard deviation from two independent experiments.

Moreover, it is interesting to note that most of the isolates collected from S9W were able to grow with 6.5% NaCl, while most of those collected from SW1 were not able to grow with this salt concentration. This might result from a selection of strains resistant to the high salt concentration during sausage processing. NaCl-sensitive strains, present at the beginning of the process, would stop growing when NaCl concentration becomes too high. This is in agreement with the hypothesis that different strain populations are developed at different stage of the fermentation process. Therefore, tolerance to NaCl is a major factor for choosing strains as starter culture in dried fermented products (Rovira et al., 1997).

3.3. Detection of the katA gene encoding catalase

Hydrogen peroxide can interfere with organoleptic properties of fermented meat products leading to rancidity and discoloration of the final product. *L. sakei* has been reported to possess a heme-dependent catalase, which can be active in meat products as these substrates contain abundant heme sources (Hertel et al., 1998). Catalase hydrolyses hydrogen peroxide (Lücke, 1985). This activity is thus considered as a desired property for starter cultures.

L. sakei isolates were screened for the presence of the *katA* gene. DNA fragments were amplified from chromosomal DNA with specific primers. For all isolates, but E1L-6, a PCR product of the expected size

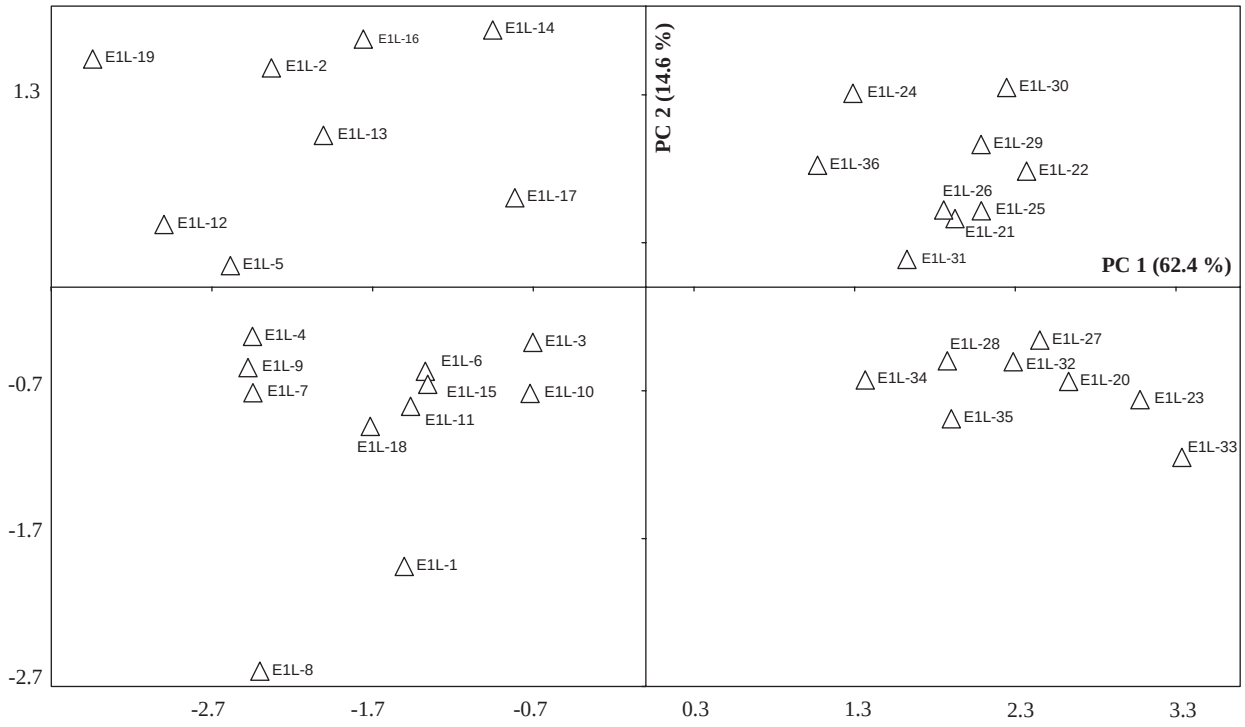

Fig. 1. Principal components analysis similarity map determined by principal components 1 and 2 for growth and acidification kinetics parameters data of *L. sakei* isolates.

(410 bp, see Fig. 2) was obtained, suggesting the presence of a *katA* gene in most strains.

3.4. Antagonistic activity against L. innocua and S. aureus

LAB originally isolated from traditional sausages are probably the best candidates for improving the microbiological safety of these products, because they are well adapted to the conditions encountered in sausages and should therefore be more competitive than LAB from other sources. We looked for antagonistic activities of *L. sakei* isolates against *L. innocua* ATCC 33090 and *S. aureus* E1S-5.

Of the 36 isolates considered, only 1 was found to exhibit antibacterial activity against *L. innocua* ATCC 33090. No isolate was able to inhibit *S. aureus* E1S-5. Similar results were obtained by Rovira et al. (1997) who showed that among 119 *L. sakei* investigated, only three species displayed inhibitory activity against *L. monocytogenes*. However, Papamanoli et al. (2003) reported that all 49 *L. sakei* investigated displayed antibacterial activity against *L. monocytogenes* and 75% against *S. aureus*.

3.5. Enzymic activities

Most *L. sakei* isolates showed leucine and valine arylamidase. However, no isolate showed cystine arylamidase and one of them displayed trypsin and α-chymotrypsin activities. The degradation of amino acids into volatile molecules plays a putative role in flavor development of dry sausage. Aldehydes, alcohols and acids, which have very low threshold values, result from the degradation of leucine, valine, phenylalanine and methionine (Montel et al., 1998). Proteolytic activity (proteinase and aminopeptidase activities) has been demonstrated for some *L. sakei* during the ripening process of fermented sausage (Fadda et al., 1999). Nevertheless, it seems difficult to establish the microbial role and the enzymic role of this accumulation of protein catabolism products (Champomier-Vergès et al., 2002).

Lipolytic activities of esterase (C4), esterase lipase (C8) and lipase (C14) were not detected for all isolates, but for E1L-31, which showed a lipase (C14) activity. These results differ with those of Papamanoli et al. (2003) who showed that 31% of the 49 *L. sakei* isolates investigated exhibited a lipolytic activity. Montel et al. (1998) reported that *Lactobacillus* species are weakly lipolytic. Moreover, bacterial lipases seem to be ineffective under conditions that prevail during fermentation of dry sausages (Demeyer et al., 2000).

Acid phosphatase and phosphohydrolase activities were shown for most isolates and only one isolate exhibited alkaline phosphatase. Similar results were reported by Papamanoli et al. (2003).

Only 16% of the isolates showed β-galactosidase activity, 3% β-glucuronidase activity, 3% α-glucosidase

Table 2
Physiological traits of *L. sakei* isolates

Isolate	Temperature				pH					[NaCl]			AA	CO_2
	0 °C	4 °C	15 °C	45 °C	3.9	4.2	5.2	9	9.6	4%	6.5%	10%		
E1L-1	–	+	+	–	–	+*	+	+	+	+	+	–	+	–
E1L-2	–	+	+	–	–	+	+	+	+	+	+	–	+	–
E1L-3	–	+	+	–	–	+	+	+	+	+	+	–	+	–
E1L-4	–	+	+	–	–	+	+	+	+	+	+	–	+	–
E1L-5	–	+	+	–	–	+	+	+	+	+	+	–	+	–
E1L-6	–	+	+	–	–	+	+	+	+	+	–	–	+	–
E1L-7	–	+	+	–	–	+	+	+	+	+	+*	–	+	–
E1L-8	–	+	+	–	–	+	+	+	+	+	–	–	+	–
E1L-9	–	+	+	–	–	+	+	+	+	+	–	–	+	–
E1L-10	–	+	+	–	–	+	+	+	+	+	+*	–	+	–
E1L-11	–	+	+	–	–	+	+	+	+	+	+	–	+	–
E1L-12	–	+	+	–	–	+	+	+	+	+	+	–	+	–
E1L-13	–	+	+	–	–	+	+	+	+	+	+	–	+	–
E1L-14	–	+	+	–	–	+	+	+	+	+	+	–	+	–
E1L-15	–	+	+	–	–	–*	+	+	+	+	+	–	+	–
E1L-16	–	+	+	–	–	+*	+	+	+	+	+	–	+	–
E1L-17	–	+	+	–	–	+	+	+	+	+	+	–	+	–
E1L-18	–	+	+	–	–	+	+	+	+	+	+*	–	+	–
E1L-19	–	+	+	–	–	+	+	+	+	+	+	–	+	–
E1L-20	–	+	+	–	–	+	+	+	+	+	–	–	+	–
E1L-21	–	+	+	–	–	+	+	+	+	+	–	–	+	–
E1L-22	–	+	+	–	+*	+	+	+	+	+	+	–	+	–
E1L-23	–	+	+	–	–	+	+	+	+	+	+	–	+	–
E1L-24	–	+	+	–	+*	+	+	+	+	+	+	–	+	–
E1L-25	–	+	+	–	+	+	+	+	+	+	–	–	+	–
E1L-26	–	+	+	–	+	+	+	+	+	+	+	–	+	–
E1L-27	–	+	+	–	–	+	+	+	+	+	–	–	+	–
E1L-28	–	–	+	–	–	+	+	+	+	+	–	–	+	–
E1L-29	–	+	+	–	+	+	+	+	+	+	–	–	+	–
E1L-30	–	+	+	–	–	+	+	+	+	+	–	–	+	–
E1L-31	–	+	+	–	–	+	+	+	+	+	+	–	+	–
E1L-32	–	+	+	–	–	+	+	+	+	+	–	–	+	–
E1L-33	–	+	+	–	–	+	+	+	+	+	–	–	+	–
E1L-34	–	+	+	–	–	+	+	+	+	+	–	–	+	–
E1L-35	–	+	+	–	–	+	+	+	+	+	+	–	+	–
E1L-36	–	+	+	–	–*	+	+	+	+	+	+	–	+	–

–: Negatif result; –*: one of the three assays is positive; +*: two of the three assays are positive; and +: positive result.

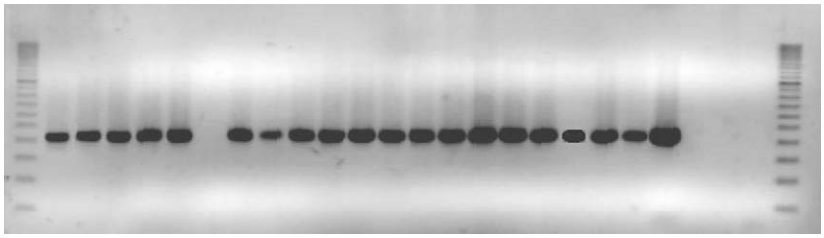

Fig. 2. PCR products obtained from *L. sakei* isolates with primers specific for the *kat*A gene (702-F/310-R) (M, 100-bp DNA molecular weight marker; lane 1, E1L-1; lane 2, E1L-2; lane 3, E1L-3; lane 4, E1L-4; lane 5, E1L-5; lane 6, E1L-6; lane 7, E1L-7; lane 8, E1L-8; lane 9, E1L-9; lane 10, E1L-10; lane 11, E1L-11; lane 12, E1L-12; lane 13, E1L-13; lane 14, E1L-14; lane 15, E1L-15; lane 16, E1L-16; lane 17, E1L-17; lane 18, E1L-18; lane 19, E1L-19; lane 20, E1L-20; lane 21, *L. sakei* CIP 103139; lane 22, *Lb. curvatus* CIP 102992; lane 23, *Lb. farciminis* CIP 103136; and lane 24, negative control).

activity, 8% *N*-acetyl-β-glucosaminidase activity, and 6% α-mannosidase and α-fucosidase activities, respectively. Similar results were reported by Papamanoli et al. (2003). It should be noted that Buckenüskes (1993) suggested that a lactose-negative phenotype is a selection criterion for LAB to be used as meat starter

cultures. However, the ability of *L. sakei* to use lactose may depend from a β-galactosidase or a phospho-β-galactosidase, the presence of which is strain dependent (Obst et al., 1992). Furthermore, it is interesting to note that all isolates did not show a melibiase activity while all, but E1L-26, showed melibiose-positive phenotype with the API 50CH gallery (data not shown) (Table 3).

3.6. Selection of *L. sakei* isolates for their use as meat starter cultures

In order to select the best *L. sakei* isolates, we incorporated into the technological parameters results data, two virtual isolates representing the 'best' and the 'worst' LAB starter culture. The results attributed to each variable for the 'best' and 'worst' starter LAB were determined on the basis of selection criteria reported by Buckenüskes (1993) and Holzapfel (2002), i.e. (1) fast growth and high growth rate, (2) fast production of lactic acid and high acidification rate, (3) ability to grow at different temperatures, (4) ability to grow at different salt concentrations, (5) ability to grow at different pH values, (6) degradation of carbohydrate by homofermentative pathway, (7) catalase positive, (8) lactose negative, (9) formation of flavor and contribution to sensory attributes and (10) antagonistic activity against pathogens.

Table 3
Enzymic activities of *L. sakei* isolates

Isolate	Enzymic activities													
	2	5	6	7	9	10	11	12	14	15	16	18	19	20
E1L-1	−	−	+	+	−	−	+	−	−	−	−	−	−	−
E1L-2	−	−	+	+	−	−	−	−	−	−	−	−	−	−
E1L-3	−	−	+	+	−	−	+	−	−	−	−	−	−	−
E1L-4	−	−	+	+	+	+	+	+	−	−	−	−	−	−
E1L−5	−	−	+	+	−	−	+	+	−	−	−	−	−	−
E1L-6	−	−	+	+	−	−	+	+	−	−	−	−	−	−
E1L-7	−	−	+	+	−	−	+	−	+	−	−	−	−	−
E1L-8	−	−	+	+	−	−	+	+	−	−	−	−	+	+
E1L-9	−	−	+	+	−	−	+	+	−	−	−	−	−	−
E1L-10	−	−	+	+	−	−	+	+	−	−	−	−	−	−
E1L-11	−	−	+	+	−	−	+	+	−	−	−	−	−	−
E1L-12	−	−	+	+	−	−	+	+	−	−	−	−	−	−
E1L-13	−	−	+	+	−	−	+	−	+	−	+	−	−	+
E1L-14	−	−	+	+	−	−	+	−	−	−	−	−	−	−
E1L-15	−	−	+	+	−	−	+	+	−	−	−	−	−	−
E1L-16	−	−	+	+	−	−	+	+	−	−	−	−	−	−
E1L-17	+	−	+	+	−	−	+	+	−	+	−	−	+	−
E1L-18	−	−	+	+	−	−	+	+	−	−	−	+	−	−
E1L-19	−	−	+	+	−	−	+	+	−	−	−	−	−	−
E1L-20	−	−	+	+	−	−	+	+	−	−	−	−	−	−
E1L-21	−	−	−	+	−	−	−	+	−	−	−	−	−	−
E1L-22	−	−	+	+	−	−	−	+	−	−	−	−	−	−
E1L-23	−	−	+	+	−	−	−	+	−	−	−	+	−	−
E1L-24	−	−	+	+	−	−	−	+	+	−	−	−	−	−
E1L-25	−	−	+	+	−	−	−	+	−	−	−	−	−	−
E1L-26	−	−	+	+	−	−	−	+	−	−	−	−	−	−
E1L-27	−	−	+	+	−	−	+	+	+	−	−	−	−	−
E1L-28	−	−	+	−	−	−	+	−	+	−	−	−	−	−
E1L-29	−	−	+	+	−	−	+	+	−	−	−	+	−	−
E1L-30	−	−	+	+	−	−	+	−	−	−	−	−	−	−
E1L-31	−	+	+	+	−	−	−	−	+	−	−	−	−	−
E1L-32	−	−	−	+	−	−	−	+	−	−	−	−	−	−
E1L-33	−	−	+	+	−	−	−	+	−	−	−	−	−	−
E1L-34	−	−	−	+	−	−	+	+	−	−	−	−	−	−
E1L-35	−	−	+	+	−	−	−	+	−	−	−	−	−	−
E1L-36	−	−	−	+	−	−	−	+	−	−	−	−	−	−

2: Alcaline phosphatase; 5: lipase (C14); 6: leucine arylamidase; 7: valine arylamidase; 9: trypsine; 10: α-chymotrypsine; 11: acid phosphatase; 12: naphtol phosphohydrolase; 14: β-galactosidase (lactase); 15: β-glucuronidase (hyaluronidase); 16: α-glucosidase (maltase); 18: *N*-acetyl-β-glucosaminidase (chitinase); 19: α-mannosidase; 20: α-fucosidase.
Enzymic activities not detected in any isolates: 3: esterase (C4); 4: esterase lipase (C8); 8: cystine arylamidase; 13: α-galactosidase (melibiase); 17: β-glucosidase (cellulase).

Therefore, considering the 'best' LAB starter culture, G_L, A_L, and pH (48 h) values attributed to it were the minimum values of those recorded for all isolates, while G_K, A_K and OD (48 h) values were the maximum ones. A positive result was attributed to tests of growth at different temperatures, pH values, salt concentrations and on AA; presence of a *katA* gene; antagonism against *L. innocua*; antagonism against *S. aureus*; leucine, valine and cystine arylamidase; esterase (C4), esterase lipase (C8) and lipase (C14). A negative result was attributed to tests of gas production from glucose and β-galactosidase. Results of the 'worst' LAB starter culture were opposed to those of the 'best' one.

To perform an AHC, weights were attributed to each variable, i.e. selection criterion, as regards the importance of the criterion in the production of fermented sausages. The primary purpose of LAB starter cultures use in meat fermentation is to enhance the product safety and to extend its shelf life (Lücke, 2000). This can be achieved by the ability of LAB to reduce the pH and to produce antimicrobial compounds, namely bacteriocins. The ability to reduce pH, which is a result of carbohydrates conversion to lactic acid, is conditioned by the ability of the lactic bacterium to grow and to be competitive in the conditions of sausage manufacturing, i.e. salt concentration, manufacturing temperature and product acidity. Salt concentration is about 2% ($a_w = 0.94-0.98$) in the batter and can reach 15% ($a_w = 0.85-0.86$) in the final product (Lücke and Hechelmann, 1987; Montel, 1999). Manufacturing temperature range from 4 to 7 °C when preparing the batter (Baracco et al., 1990) from 18 to 24 °C during the fermentation period (Montel, 1999) and from 12 to 15 °C during the drying period (Montel, 1999). The initial pH of the batter, which is generally about 6, decreases during the fermentation and reaches a values between 4.6 and 5.1. Thereafter, yeast, namely *Debaromyces hansenii*, regulates the acidity of the product (Cook, 1995) and the final pH ranges from 5.1 to 5.5 (Montel, 1999). Thus, the growth and acidification rates and lag phases and the growth at 4, 15 °C, pH 4.2, 5.2 and with 4%, 6.5% and 10% are limiting factors in the starter culture selection, a weight of 1 was attributed to each variable.

As mentioned above, safety improvement and shelf life extension can also be achieved by the production of bacteriocins. However, their influence in sausage appears to be limited because they are often bound to the matrix and are degraded by tissue proteases (Schillinger et al., 1996). Thus, antagonism against *L. innocua* and *S. aureus* was not a limiting factor but appreciable; a weight of 0.5 was attributed to each variable.

The *katA* gene, the homofermentative profile and the lactose-negative phenotype are factors improving the organoleptic quality of sausages; a weight of 0.5 was attributed to each variable.

As LAB are less proteolytic and only weakly lipolytic, in sausages, compared to catalase-positive cocci

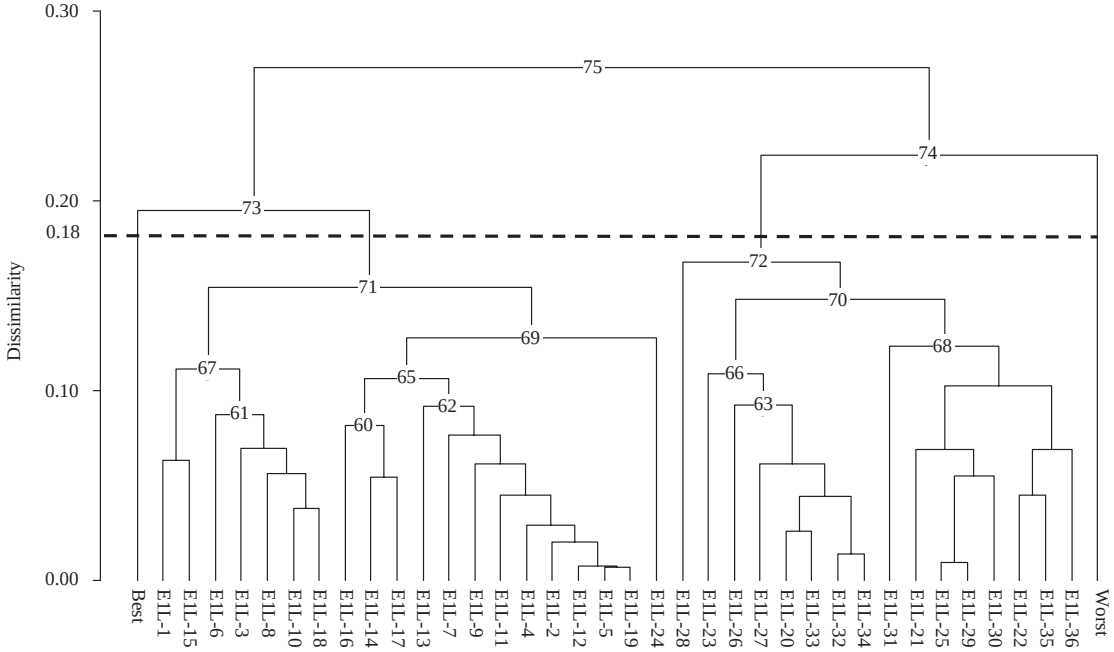

Fig. 3. Hierarchical clustering performed on the technological parameters data. Dashed bar shows level of statistical significance of the discrimination.

Table 4
Dissimilarity matrix of observations forming the node 73 (clusters 1 and 2; Fig. 3)

	Best	E1L-1	E1L-2	E1L-3	E1L-4	E1L-5	E1L-6	E1L-7	E1L-8	E1L-9	E1L-10	E1L-11	E1L-12	E1L-13	E1L-14	E1L-15	E1L-16	E1L-17	E1L-18	E1L-19	E1L-24
Best	0	0.61	0.52	0.63	0.53	0.52	0.60	0.59	0.62	0.55	0.63	0.58	**0.51**	0.62	0.58	0.58	0.58	0.58	0.61	**0.51**	0.59
E1L-1	0.61	0	0.21	0.11	0.14	0.17	0.20	0.24	0.15	0.21	0.10	0.11	0.18	0.29	0.22	0.06	0.18	0.21	0.12	0.18	0.34
E1L-2	0.52	0.21	0	0.13	0.06	0.04	0.21	0.13	0.20	0.13	0.15	0.11	0.03	0.12	0.07	0.16	0.11	0.08	0.14	0.03	0.25
E1L-3	0.63	0.11	0.13	0	0.11	0.13	0.17	0.21	0.11	0.17	0.06	0.07	0.14	0.20	0.13	0.11	0.21	0.13	0.08	0.14	0.28
E1L-4	0.53	0.14	0.06	0.11	0	0.03	0.21	0.12	0.16	0.08	0.12	0.05	0.03	0.15	0.08	0.14	0.15	0.12	0.13	0.04	0.26
E1L-5	0.52	0.17	0.04	0.13	0.03	0	0.19	0.10	0.17	0.10	0.13	0.07	0.01	0.13	0.09	0.13	0.12	0.10	0.11	0.01	0.28
E1L-6	0.60	0.20	0.21	0.17	0.21	0.19	0	0.22	0.07	0.14	0.12	0.17	0.19	0.29	0.27	0.20	0.30	0.23	0.10	0.19	0.40
E1L-7	0.59	0.24	0.13	0.21	0.12	0.10	0.22	0	0.19	0.14	0.17	0.15	0.10	0.09	0.19	0.21	0.21	0.20	0.15	0.11	0.24
E1L-8	0.62	0.15	0.20	0.11	0.16	0.17	0.07	0.19	0	0.09	0.07	0.12	0.17	0.28	0.23	0.16	0.28	0.21	0.06	0.17	0.37
E1L-9	0.55	0.21	0.13	0.17	0.08	0.10	0.14	0.14	0.09	0	0.13	0.11	0.09	0.21	0.15	0.21	0.21	0.17	0.15	0.10	0.31
E1L-10	0.63	0.10	0.15	0.06	0.12	0.13	0.12	0.17	0.07	0.13	0	0.07	0.14	0.24	0.17	0.13	0.24	0.16	0.04	0.14	0.31
E1L-11	0.58	0.11	0.11	0.07	0.05	0.07	0.17	0.15	0.12	0.11	0.07	0	0.08	0.19	0.12	0.14	0.19	0.11	0.08	0.08	0.26
E1L-12	**0.51**	0.18	0.03	0.14	0.03	0.01	0.19	0.10	0.17	0.09	0.14	0.08	0	0.13	0.09	0.14	0.12	0.11	0.11	0.01	0.28
E1L-13	0.62	0.29	0.12	0.20	0.15	0.13	0.29	0.09	0.28	0.21	0.24	0.19	0.13	0	0.10	0.23	0.14	0.12	0.23	0.13	0.15
E1L-14	0.58	0.22	0.07	0.13	0.08	0.09	0.27	0.19	0.23	0.15	0.17	0.12	0.09	0.10	0	0.20	0.09	0.05	0.20	0.09	0.19
E1L-15	0.58	0.06	0.16	0.11	0.14	0.13	0.20	0.21	0.16	0.21	0.13	0.14	0.14	0.23	0.20	0	0.13	0.21	0.11	0.14	0.37
E1L-16	0.58	0.18	0.11	0.21	0.15	0.12	0.30	0.21	0.28	0.21	0.24	0.19	0.12	0.14	0.09	0.13	0	0.10	0.23	0.12	0.27
E1L-17	0.58	0.21	0.08	0.13	0.12	0.10	0.23	0.20	0.21	0.17	0.16	0.11	0.11	0.12	0.05	0.21	0.10	0	0.16	0.10	0.18
E1L-18	0.61	0.12	0.14	0.08	0.13	0.11	0.10	0.15	0.06	0.15	0.04	0.08	0.11	0.23	0.20	0.11	0.23	0.16	0	0.12	0.34
E1L-19	**0.51**	0.18	0.03	0.14	0.04	0.01	0.19	0.11	0.17	0.10	0.14	0.08	0.01	0.13	0.09	0.14	0.12	0.10	0.12	0	0.28
E1L-24	0.59	0.34	0.25	0.28	0.26	0.28	0.40	0.24	0.37	0.31	0.31	0.26	0.28	0.15	0.19	0.37	0.27	0.18	0.34	0.28	0

(*Staphylococcus xylosus* and *Staphylococcus carnosus*) (Montel et al., 1998), a weight of 0.25 was attributed to each variable.

The OD (48 h) and pH (48 h) values and the growth at 0, 45 °C, pH 3.9, 9 and 9.6 are not limiting factors but appreciable characters; a weight of about 0.1 was attributed to each variable.

As data were presented in quantitative and qualitative format in which correlation between variables was non-parametric, the analysis consisted of normalized non-parametric (Kendall) AHC using a strong linkage as aggregation method. When the increase in dissimilarity was strong, an automatic truncation stopped aggregating observations.

Fig. 3 presents the dendrogram obtained. Observations were clustered in four groups following a truncation at 0.18 of dissimilarity level. Group 1 encompassed by the 'best' LAB starter culture. Group 2 comprised the S9W isolates and the E1L-24 isolate. Group 3 contained the remaining S1W isolates and that from the knife. Finally, group 4 corresponded to the 'worst' LAB starter culture. Group 2 was not very distant from the first group, since both depend on node 73; but the difference remains noticeable. Alternatively, groups 3 and 4 depend on node 74. Thus isolates forming group 2 were less distant from the 'best' starter culture compared to those of group 3.

Considering groups 1 and 2, the dissimilarity matrix obtained (Table 4) showed that *L. sakei* isolates E1L-12 and E1L-19 were the less distant isolates from the 'best' starter culture. Indeed, they showed the lowest dissimilarity level (0.51) from the 'best' starter. E1L-2 and E1L-5 were also less distant showing dissimilarity levels of about 0.52.

These results indicate that *L. sakei* E1L-12 and/or E1L-19 could be used in the elaboration of starter cultures for this small-scale facility. A further investigation has to be performed to investigate the behavior of E1L-12, E1L-19, E1L-2 and E1L-5 in the presence of a selected coagulase-negative staphylococci, namely *S. equorum* also isolated from the same small-scale facility. Indeed, Morot-Bizot (2005) showed that *S. equorum* was the dominant catalase-positive cocci isolated from the same studied small-scale facility. This bacterium has been already reported as starter culture in cheese manufacturing (Place et al., 2003). Therefore, as technological flora, both the lactic bacterium and the catalase-positive cocci should coexist without antagonism. Challenge tests have to be also performed to investigate the behavior of these isolates as starter cultures in vivo.

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