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Selection of *Lactobacillus* strains from fermented sausages for their potential use as probiotics

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Abstract

A rapid screening method was used to isolate potentially probiotic *Lactobacillus* strains from fermented sausages after enrichment in MRS broth at pH 2.5 followed by bile salt stressing (1% bile salts w/v). One hundred and fifty acid- and bile-resistant strains were selected, avoiding preliminary and time-consuming isolation steps. Strains were further characterized for survival at pH 2.5 for 3 h in phosphate-buffered saline and for growth in the presence of 0.3% bile salts with and without pre-exposure at low pH. Twenty-eight strains showed a survival >80% at pH 2.5 for 3 h; moreover, most of the strains were able to grow in the presence of 0.3% bile salts. Low pH and bile resistance was shown to be dependent on both the species, identified by phenotypic and molecular methods, and the strain tested.

This is the first report on the direct selection of potentially probiotic lactobacilli from dry fermented sausages. Technologically interesting strains may be used in the future as probiotic starter cultures for novel fermented sausage manufacture.

Keywords: *Lactobacillus*; Probiotics; Dry fermented sausages; Acid tolerance; Bile tolerance; Starter cultures

1. Introduction

The relationship between certain foods and health benefits has been investigated for many years. These studies have allowed the development of the concept of a functional food as a food or food ingredient with positive effects on host health and/or well-being beyond its nutritional value (Huggett & Verschuren, 1996). In more recent years, attention has been directed towards food additives that may exert a positive effect on the microflora of the human gastrointestinal tract and, therefore, towards probiotics (Ziemer & Gibson, 1998).

The acid and bile tolerances are two fundamental properties that indicate the ability of a probiotic microorganism to survive the passage through the gastrointestinal tract, resisting the acidic conditions in the stomach and the bile acids at the beginning of the small intestine (Hyronimus, Le Marrec, Hadj Sassi, & Deschamps, 2000; Park, Lee, Kim, & Shin, 2002; Prasad, Gill, Smart, & Gopal, 1998).

The survival of bacteria in the gastric juice depends on their ability to tolerate low pH. The pH of excreted HCl in the stomach is 0.9, but the presence of food raises the pH value to 3.0 (Erkkila & Petaja, 2000). After food ingestion, it takes 2–4 h for the stomach to empty. For those bacteria that survive the environmental conditions of the stomach, the further challenge is bile secretion and bile salts in the *duodenum*. The bile salts are released into the upper small intestine after ingestion of fatty meals and they have a detergent-like function. Since the cell membranes of microorganisms are composed of lipids and fatty acids, the bile salts are critical to them. However, some microorganisms are able to reduce this detergent effect by their ability to hydrolyse bile salts by bile salt hydrolase enzyme (BSH) and thus to decrease their solubility (Erkkila & Petaja, 2000). BSH activity has been found in many genera including *Lactobacillus* (Gilliland & Speck, 1977). Bile salts resistance varies a

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lot between the *Lactobacillus* species and also between strains, and the mechanism is still unknown (Erkkila & Petaja, 2000). Although the bile concentration of the gastrointestinal tract varies, the mean intestinal bile concentration for the screening of a resistant probiotic strain is believed to be 0.3% w/v (Gilliland, Staley, & Bush, 1984).

The most common use of probiotic microorganisms is in fermented dairy products (Ouwehand, Salminen, & Isolauri, 2002), although recently a new idea is developing to use probiotic strains in other foods such as fermented meat products (Hugas & Monfort, 1997; Hammes & Hertel, 1998; Tyopponen, Petaja, & Mattila-Sandholm, 2003). In fact, the meat industry has begun to find novel starter cultures with additional value (Erkkila et al., 2001a). Many scientists have recently proposed the use of probiotic meat starter cultures for dry fermented sausage manufacture (Hugas & Monfort, 1997; Incze, 1998). It has been also demonstrated that it is possible to successfully use these probiotics as starter cultures for dry fermented sausages, since there are no significant technological and sensorial differences between the sausages fermented by probiotics or non-probiotics (Erkkila et al., 2001a; Erkkila, Suihko, Eerola, Petaja, & Mattila-Sandholm, 2001b).

Some studies describe the behaviour of previously selected probiotic strains for their use in the sausages production (Erkkila et al., 2001b) or the potential probiotic use of some selected strains present in commercial meat starter cultures (Erkkila et al., 2001a). Technologically interesting strains to use as starters are usually selected from the food products they are going to be employed for. Therefore, to determine the potential to use probiotics in meat products, a screening procedure was performed to select potentially probiotic *Lactobacillus* strains directly from fermented meat products.

2. Materials and methods

2.1. Isolation and screening of bacterial strains from fermented sausages

Ten samples (5 g each) of traditional dry fermented sausages (Table 1) were inoculated into 45 ml of MRS broth (OXOID Ltd., Basingstoke, Hampshire, England). After anaerobic incubation at 37 °C for 12–15 h (anaerobic jar and Anaero*Gen*^{TM}, OXOID), 10 ml of the broth were centrifuged at 1000*g* for 15 min and the pellet was resuspended into 10 ml of MRS broth adjusted to pH 2.5 with HCl (5 mol L^{-1}). After 3 and 24 h of anaerobic incubation at 37 °C, 5 ml of the incubation medium were centrifuged at 9000*g* for 15 min and the pellet resuspended in 5 ml of MRS broth containing 1% (w/v) bile salts (Bile, Sigma Chemical Co., St. Louis, MO, catalogue no. B-8381). After 2 h at 37 °C, 0.1 ml of the culture were spread plated on MRS agar medium and the plates were incubated under anaerobic conditions at 37 °C for 48–72 h. Colonies characterized by different morphologies were isolated and purified on MRS agar medium. All the isolates were examined by microscopy, gram staining and catalase reaction. Gram positive and catalase negative rods were maintained in MRS broth with 25% sterile glycerol and stored at −80 °C.

2.2. Acid tolerance

Cultures were grown in MRS broth at 37 °C for 24 h. A 0.5 ml aliquot of the bacterial culture was inoculated into 10 ml of phosphate-buffered saline (PBS) adjusted to pH values of 6.0 and 2.5 with 5 mol L^{-1} HCl. PBS was prepared by dissolving NaCl (9 g L^{-1}), $Na_2HPO_4 \cdot 2H_2O$ (9 g L^{-1}) and KH_2PO_4 (1.5 g L^{-1}) in distilled water, according to Erkkila & Petaja (2000). The initial bacterial concentration was 10^6–10^8 CFU ml^{-1}. Samples were incubated for 3 h at 37 °C. Cells were serially diluted 10-fold in Ringer solution (OXOID) and the residual viable population was determined by plate counting on MRS agar after 48–72 h of incubation under anaerobic conditions. The survival rate was calculated as the percentage of colonies grown on MRS agar compared to the initial bacterial concentration.

2.3. Bile tolerance

The minimal inhibitory concentration (MIC) of bile was determined on MRS agar plates containing bile salts (0–4.0% w/v), before and after pre-exposure to low pH (see Section 2.2), by spotting 10 μl of overnight cultures (about 1×10^6 CFU). Plates were incubated at 37 °C under anaerobic conditions for 4 days. The MIC was defined as the lowest concentration totally inhibiting the growth as evaluated by examining the spots.

The method described by Gilliland et al. (1984) was used to determine bile tolerance. Isolates were grown in 20 ml of MRS broth at 37 °C for 24 h. Then 0.2 ml of the cultures were inoculated into 20 ml of MRS broth prepared with and without 0.3% bile salts. The initial inoculum concentration was 10^5–10^6 CFU ml^{-1}, and all samples were incubated for 24 h at 37 °C. Growths of control (no bile) and test cultures (0.3% bile salts) were monitored after 0, 2, 4, 8 and 24 h of incubation by measuring the absorbance at 600 nm using a spectrophotometer (Beckman, DU 640).

To determine bile tolerance of strains after pre-exposure to low pH, each strain was grown in MRS broth at 37 °C for 24 h. Cells were harvested by centrifugation (9000*g*, 15 min) and resuspended into PBS adjusted to pH 2.5, prepared as described above. After incubation for 3 h at 37 °C, cells were harvested by centrifugation (9000*g*, 15 min) and resuspended into MRS broth pre-

Table 1
Origin and number of *Lactobacillus* isolates selected after screening for tolerance to acid and to bile salts

Sausage[a]	No. of isolates		No. of isolates from procedure Y[c] surviving (%) at pH 2.5 for 3 h			
	X[b]	Y[c]	<60	60 ⩽ % < 70	70 ⩽ % < 80	⩾ 80%
A	5	5	0	1	0	4
B	7	7	3	2	1	1
C	12	11	1	2	0	8
D	7	6	2	0	1	3
E	10	8	1	2	2	3
F	8	7	2	0	2	3
G	2	4	1	0	0	3
H	4	10	5	1	2	2
I	13	8	6	1	0	1
J	9	7	5	2	0	0
Total of isolates	77	73	26	11	8	28

[a] A, Salame "Tipo Milano" (producer 1); B, Salame "Tipo Milano" (producer 2); C, Salame "Tipo Napoli" Capua (producer 3); D, Salame "Tipo Napoli" (producer 1); E, Salame "Tipo Napoli" (producer 2); F, Salame "Tipo Napoli" Portici (producer 4); G, Salsiccia di Capua (Caserta–Campania region); H, Soppressata di Formicola (Caserta–Campania region); I, Soppressata di Gioi (Salerno–Campania region); J, Salsiccia di Frascineto (Cosenza–Calabria region).
[b] X – 3 h at pH 2.5 followed by 2 h in presence of 1% bile salts.
[c] Y – 24 h at pH 2.5 followed by 2 h in presence of 1% bile salts.

pared with and without 0.3% bile salts. Growth of control (no bile) and test cultures (0.3% bile salts) were checked after 0, 1, 2, 3, 4, 20, 21, 22, 23, 24, 26 and 28 h of incubation by measuring absorbance at 600 nm.

2.4. Identification of the isolates

Lactobacillus strains were identified by using API 50 CHL (bio-Mérieux, Marcy l'Etoile, France) following the manufacturer's instructions.

DNA extraction was carried out from an overnight culture in MRS broth. Two milliliters of the broth was centrifuged at 22,000*g* for 10 min and the pellet was washed once with 1 ml of ET (50 mmol L^{-1} EDTA – 5 mmol L^{-1} Tris–HCl). After centrifugation, the pellet was resuspended in 250 μl of ET containing lysozime (3 mg ml^{-1}, Sigma Chemical Co., St. Louis, MO) and RNAse (0.4 mg ml^{-1}, Sigma) and incubated overnight at 37 °C. Successively, 25 μl of 25% SDS (Sigma) and 2 μl of Pronase E (20 mg ml^{-1}, Sigma) were added and the mixture was hand-shaken by inverting the tube. After incubation at 37 °C for 1 h, the sample was cooled at 4 °C for 15 min and 1 vol. of ammonium acetate 5 mol L^{-1} was added to the sample which was then centrifuged at 36,000*g* for 15 min at 4 °C. The supernatant was precipitated with 2 vol. of ice-cold ethanol and, after incubation at −20 °C for 30 min, centrifuged at 36,000*g* for 15 min at 4 °C. Finally, the pellet was dried and resuspended in 50 μl of DNA Rehydration Solution (Wizard DNA purification kit, Promega, Madison, WI) by incubation at 55 °C for 60 min.

Synthetic oligonucleotide primers described by Weisburg, Barns, Pelletier, and Lane (1991) fD1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and rD1 (5′-AAG GAG GTG ATC CAG CC-3′) (*Escherichia coli* positions 8–17 and 1540–1524, respectively) were used to amplify the 16S rDNA. PCR mixture was prepared as previously described (Ercolini, Moschetti, Blaiotta, & Coppola, 2001), while PCR conditions consisted of 30 cycles (1 min at 94 °C, 45 sec at 54 °C, 2 min at 72 °C) plus one additional cycle at 72 °C for 7 min as a final chain elongation.

The presence of PCR products was verified by agarose (1.5% w/v) gel electrophoresis, at 7 V cm^{-1} for 2 h, purified by using a QIAquick PCR purification kit (Qiagen S.p.A., Milan) and sequenced by using the primer 5′-TAA TGG CGC CGA CGA CC-3′ (*E. coli* positions 517–533) complementary to primer v3r described by Muyzer, De Waal, and Uitterlinden (1993). The DNA sequences were determined by the dideoxy chain termination method by using the DNA sequencing kit (Perkin–Elmer Cetus, Emeryville, CA) according to the manufacturer's instructions. The sequences were analyzed by MacDNasis Pro v3.0.7 (Hitachi Software Engineering Europe S.A., Olivet Cedex) and research for DNA similarity was performed with the GenBank and EMBL database (Altschul et al., 1997). The GenBank accession numbers of the sequences are reported in Table 3.

3. Results

3.1. Screening of acid- and bile-resistant strains and determination of acid tolerance

The screening at low pH (2.5) and in presence of bile salts (1% w/v) used in this study led to the isolation of

150 resistant strains of *Lactobacillus* spp. from 10 different fermented sausages, as reported in Table 1. The 73 strains selected at low pH and in presence of bile salts after incubation for 24 h were further tested in PBS adjusted to pH 6.0 and pH 2.5 after 3 h of incubation at 37 °C. All the strains showed 100% survival at pH 6.0, without any decrease in viable cell numbers (data not shown).

The results obtained by exposure in PBS at pH 2.5 for 3 h at 37 °C are reported in Table 1. Only sample J did not allow the isolation of any strain showing survival >80%. On the basis of survival percentage (%) it was possible to distinguish four groups of isolates. Twenty-six strains showed a survival percentage <60%, 11 strains $6 \leqslant \% < 70$, 8 strains $70 \leqslant \% < 80$ and finally 28 strains $\geqslant 80\%$. Only the 28 strains reaching a bench mark of 80% survival after 3 h of incubation at pH 2.5 were further considered. In Fig. 1 differences in survival at pH 2.5 of the 28 strains are reported. Only 50% of the strains displayed a survival >100% although all of them showed an increase of <1 log CFU ml^{-1}.

3.2. Bile tolerance

Determination of MIC was applied to determine the strains capable of withstanding a bile concentration above 0.3%, with and without pre-exposure in PBS at pH 2.5 for 3 h (Table 2). For 25 *Lactobacillus* strains (89.3%), the MIC could not be determined, since they were able to grow on MRS agar containing up to 4.0% bile salts, if not pre-exposed to low pH. As the bile salts concentration increased, the growth of the spots on the agar became less homogeneous and many separated little colonies could be distinguished, showing an increasing inhibitory effect of bile salts on the growth capability. On the contrary, two strains, CL3 and CL8, had a MIC of 1.8% bile salts, while 1 strain, HL7, had a MIC of 1.0% bile salts.

After pre-exposure in PBS at pH 2.5 for 3 h, 19 *Lactobacillus* strains (67.8%) were able to grow on MRS agar containing up to 4.0% bile salts (Table 2). Again, as the bile salts concentration increased, the growth of the spots on the agar became less homogeneous and many separated little colonies could be distinguished.

The bile tolerance of the 28 selected *Lactobacillus* strains which presented a survival % in PBS at pH 2.5 for 3 h at 37 °C of $\geqslant 80\%$, was investigated. Each strain was inoculated in MRS broth without bile salts (control) and MRS broth containing 0.3% bile salts and the growth capability was monitored by measuring the absorbance at 600 nm at several times of incubation. The respective control of each strain showed good growth during the 24 h of incubation, reaching OD_{600} values >1.1, with the exception of the two strains CL2 and HL7 that reached OD_{600} values of 0.98 and 0.89, respectively. All the strains were able to grow in MRS broth supplemented with 0.3% bile salts, but this concentration had an inhibitory effect on their growth capability after 24 h of incubation at 37 °C and the four strains AL5, CL5, DL2 and DL6 were the most resistant, while the strain HL9 was the most sensitive.

The ability of the 28 selected *Lactobacillus* strains to grow after pre-exposure in PBS at pH 2.5 was investigated. Each strain was preincubated in PBS at pH 2.5 for 3 h at 37 °C, before resuspending in MRS broth without bile salts (control) and in MRS broth containing 0.3% bile salts. The growth capability under these conditions was monitored by measuring the absorbance at 600 nm at several times of incubation. The respective control of each strain showed a good growth during the 28 h of incubation, reaching OD_{600} values >1.0. Twenty-seven strains were able to grow in MRS broth supplemented with 0.3% bile salts, but in this experiment

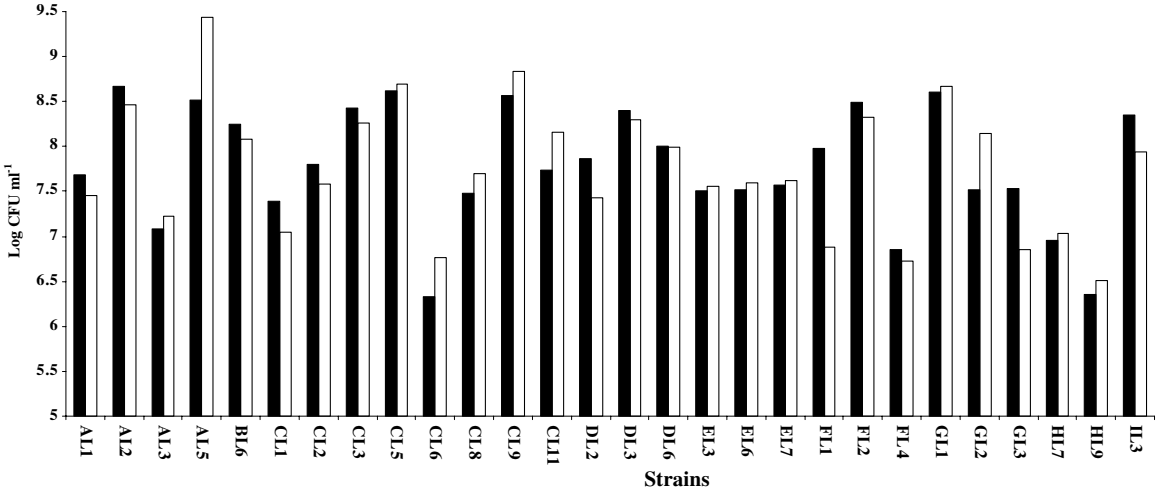

Fig. 1. Effect of the exposure in PBS at pH 2.5 for 0 h (■) and 3 h (□) on the survival of 28 selected *Lactobacillus* strains.

Table 2
Minimal inhibitory concentration of selected *Lactobacillus* strains with and without pre-exposure in PBS at pH 2.5 for 3 h at 37 °C

Strains[a]	% of bile salts																					
	0		0.3		0.5		0.7		1.0		1.2		1.4		1.6		1.8		2.0		2.5		3.0		4.0	
	1[b]	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
AL1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–
AL3	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–
CL1	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–
CL3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–
CL8	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–
GL2	+	+	+	+	+	+	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–
HL7	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
HL9	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–
IL3	+	+	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–

[a] Only the strains exhibiting MIC value <4% are reported.
[b] Growth of *Lactobacillus* strains without (1) or with (2) pre-exposure in PBS at pH 2.5 for 3 h at 37 °C. +: growth; –: no growth.

this concentration exerted inhibitory effect on their growth capability after 28 h of incubation at 37 °C. Only the strain IL3 was unable to grow in MRS broth containing 0.3% bile salts, as confirmed by the MIC determination after pre-exposure to pH 2.5. The progress of growth of the eight strains AL1, AL2, AL3, BL6, CL1, CL2, CL3 and GL2 was remarkable. In fact, they showed a very long lag phase, significantly increasing their OD_{600} values after about 20–22 h of incubation. The strain CL6 was the most resistant, whereas the strain HL7 showed a very low increase in OD_{600} value after 28 h of incubation. This strain was already among the least resistant in MRS broth with 0.3% bile salts without pre-exposure to low pH. The strains CL2, CL8 and HL9 were also inhibited by bile salts; HL9 showed a low growth in the previous experiment without pre-exposure to low pH.

3.3. Biochemical and molecular identification of selected Lactobacillus strains

To achieve preliminary identification of the 28 selected strains at the genus and species level, the isolates were subjected to sugar fermentation pattern analysis using API 50CHL test strips (Table 3). Seven isolates were identified as belonging to the species *Lactobacillus* (*Lact.*) *brevis*, 10 as *Lact. plantarum*, three as *Lact. pentosus,* six as *Lact. paracasei* subsp. *paracasei*, one as *Lact. fermentum* and one as *Lact. curvatus.*

The results obtained by molecular identification via 16S rDNA partial sequencing are reported in Table 3. The seven isolates identified by API 50CHL test as *Lact. brevis*, were confirmed by molecular identification as belonging to the same species. Ten *Lact. plantarum* and three *Lact. pentosus* isolates could be identified by API 50CHL, while the same isolates were identified as *Lact. plantarum*-group (*Lact. plantarum*, *Lact. pentosus* and *Lact. paraplantarum*) by 16S rDNA sequencing. The same result was obtained for the six isolates identified as *Lact. paracasei* subsp. *paracasei*: the molecular identification allowed us to include them within the *Lact. casei*-group (*Lact. casei*, *Lact. paracasei* and *Lact. zeae*). The strain HL9 was identified by the API 50CHL test as *Lact. fermentum*, while, by 16S rDNA partial sequencing, it was identified as *Lact. reuteri*. The strain IL3, identified by biochemical methods as *Lact. curvatus*, resulted to be either *Lact. curvatus* or *Lact. sakei*, with the same percentage of 99% by 16S rDNA sequencing.

4. Discussion

A consensus is emerging that a stringent selection criteria for identification of probiotic strains is required to achieve positive probiotic effect (Collins, Thornton, &

Table 3
Identification of *Lactobacillus* strains by API 50 CHL and 16S rDNA sequencing

Strains[a]	Identification			
	API 50 CHL	Quality of identification[b]	16S rDNA sequencing	Accession numbers
AL1	*Lact. brevis*	Good	*Lact. brevis* 99%	AY318799
AL2	*Lact. plantarum*	Very good	*Lact. plantarum, pentosus, paraplantarum* 99%	AY318800
AL3	*Lact. brevis*	Good	*Lact. brevis* 98%	AY318801
AL5	*Lact. plantarum*	Excellent	*Lact. plantarum, pentosus, paraplantarum* 98%	AY318802
BL6	*Lact. brevis*	Very good	*Lact. brevis* 98%	AY318803
CL1	*Lact. brevis*	Very good	*Lact. brevis* 96%	AY318804
CL2	*Lact. brevis*	Very good	*Lact. brevis* 98%	AY318805
CL3	*Lact. paracasei* ssp . *paracasei*	Good	*Lact. casei, paracasei, zeae* 99%	AY318806
CL5	*Lact. plantarum*	Excellent	*Lact. plantarum, pentosus, paraplantarum* 99%	AY318807
CL6	*Lact. plantarum*	Very good	*Lact. plantarum, pentosus* 98%	AY318808
CL8	*Lact. paracasei ssp. paracasei*	Very good	*Lact. casei, paracasei, zeae* 98%	AY318809
CL9	*Lact. plantarum*	Excellent	*Lact. plantarum, pentosus, paraplantarum* 99%	AY318810
CL11	*Lact. plantarum*	Excellent	*Lact. plantarum, pentosus, paraplantarum, arizonensis* 98%	AY318811
DL2	*Lact. brevis*	Very good	*Lact. brevis* 98%	AY318812
DL3	*Lact. plantarum*	Excellent	*Lact. plantarum, pentosus, paraplantarum* 98%	AY318813
DL6	*Lact. plantarum*	Excellent	*Lact. plantarum, pentosus, paraplantarum* 99%	AY318814
EL3	*Lact. paracasei* ssp. *paracasei*	Very good	*Lact. casei, paracasei, zeae* 98%	AY318815
EL6	*Lact. paracasei* ssp . *paracasei*	Very good	*Lact. casei, paracasei, zeae* 98%	AY318816
EL7	*Lact. paracasei* ssp. *paracasei*	Very good	*Lact. casei, paracasei, zeae* 97%	AY318817
FL1	*Lact. pentosus*	Excellent	*Lact. plantarum, pentosus, paraplantarum* 98%	AY318818
FL2	*Lact. pentosus*	Excellent	*Lact. plantarum, pentosus, paraplantarum* 99%	AY318819
FL4	*Lact. pentosus*	Excellent	*Lact. plantarum, pentosus, paraplantarum* 99%	AY318820
GL1	*Lact. plantarum*	Very good	*Lact. plantarum, pentosus, paraplantarum, arizonensis* 96%	AY318821
GL2	*Lact. plantarum*	Good at genus level	*Lact. plantarum, pentosus, paraplantarum* 99%	AY318822
GL3	*Lact. brevis*	Very good	*Lact. brevis* 96%	AY318823
HL7	*Lact. paracasei* ssp. *paracasei*	Doubt profile	*Lact. casei, paracasei, zeae* 99%	AY318824
HL9	*Lact. fermentum*	Good	*Lact. reuteri* 99%	AY318825
IL3	*Lact. curvatus*	Very good at genus level	*Lact. curvatus, sakei* 99%	AY318826

[a] The first letter is referred to sausage sample (see Table 1).
[b] Comments on the quality of identification derived from the ID% and the T index of the selected taxon.

Sullivan, 1998). This study was aimed to use a procedure for isolation at low pH and in presence of bile salts of potentially probiotic *Lactobacillus* strains directly from fermented sausages.

The screening method used is effective and rapid and allowed us to select 150 acid- and bile-resistant strains directly from the food sample, avoiding preliminary and time consuming isolation of the strains. After this step, isolates resistant to pH 2.5 for 24 h followed by 2 h with 1% of bile salts were further analysed for their specific tolerance to low pH and the presence of bile salts.

Several in vitro assays have been described to select acid resistant strains, i.e., exposure to pH-adjusted PBS (Conway, Gorbach, & Goldin, 1987; Park et al., 2002), incubation in gastric contents (Conway et al., 1987; Fernandez, Boris, & Barbes, 2003) and use of a dynamic model of the stomach (Marteau, Minekus, Havenaar, & Huis In't Veld, 1997). Conway et al. (1987) found survival of lactobacilli to be slightly lower when PBS was used rather than gastric juice, because components in the gastric juice may confer some protective effect on the bacterial cell. They suggested to use PBS at the desired pH to screen strains for their ability to maintain viability in vivo when exposed to gastric juice. Moreover, the probiotic strains could be buffered by food or other carrier matrix molecules following consumption and are thus not likely to be exposed to the pH of the stomach (Prasad et al., 1998). This is the reason why we used the PBS in vitro assay to test the strains previously isolated at low pH in the presence of bile salts for their resistance to pH 2.5. We were able to select 28 *Lactobacillus* strains from 10 different fermented sausages with a survival percentage $\geqslant 80\%$ when incubated in PBS at pH 2.5 for 3 h. Particularly, 14 strains showed a survival $>100\%$, seven of which belonged to *Lact. plantarum*-group and five to *Lact. casei*-group.

Acid-tolerance is a fundamental property that indicates the ability of probiotic microorganisms to survive passage through the stomach (Prasad et al., 1998; Park et al., 2002). Prasad et al. (1998), in a screening study on 200 lactic acid bacterial strains, selected four resistant strains (three of dairy origin and one of human origin) able to survive at pH 3.0 for 3 h with a survival percentage $>80\%$. When the pH value employed for in vitro

assays is very acid, small variations in pH (from 3.0 to 2.5) may considerably change the acid-tolerance of the strains tested. The pH value (2.5) used in this study for the selection of potentially probiotic strains is very selective and even though it is not the most common pH value of the human stomach it assures the isolation of the very acid-tolerant strains. It was observed that pH 2.0 is sometimes too selective and does not allow *Lact. acidophilus* to grow (Gupta, Mital, & Garg, 1996) even though growth can be observed at pH 3.0 (Gupta et al., 1996; Suskovic, Brkic, Matosic, & Maric, 1997). Xanthopoulos, Litopoulou-Tzanetaki, and Tzanetakis (2000), testing 20 *Lactobacillus* strains isolated from infant faeces, found that three *Lact. paracasei* subsp. *paracasei* strains and one *Lact. rhamnosus* strain remained almost unaffected by the low pH. Only a few studies report on the acid-resistance of lactic acid bacteria isolated from fermented sausages, Erkkila and Petaja (2000) found one *Lact. curvatus* and two *Pediococcus* spp. from commercial meat starter cultures, were resistant to pH 3.0.

The 28 strains selected on the basis of their acid-tolerance were identified by both phenotypic and molecular methods. Our results demonstrate the agreement between the two procedures only for the identification of *Lact. brevis*. In all the other cases the two types of identification did not yield exactly the same results; this was also reported by Annuk et al. (2003). In many cases the molecular identification by sequencing of the 16S rDNA allowed us to only link the strains to a group of species (i.e., *Lact. plantarum*-group). The difficulty of correct identification of these species and the increasing interest in some of their properties, such as their probiotic activities, indicates the need for a simple and reliable molecular method for their definitive differentiation to avoid false declarations of species used in probiotic products and an uncritical selection of the potentially probiotic strains.

Probiotic lactic acid bacteria are widely used in dairy but not in meat products. Before consuming, many meat products are heated and probiotic bacteria may be killed. One exception is dry sausages that are processed by fermenting, without heat (Erkkila et al., 2001b). Lactobacilli are of utmost importance in meat fermentation because of their ability to provide, in the presence of fermentable sugars, rapid and effective acidification thus preserving the sausages from the development of spoilage and pathogenic bacteria. This is why they are often used as starters in dry fermented sausage production. Combining probiotic potential and technological performances of *Lactobacillus* strains would lead to interesting probiotic starters for use in novel dry fermented sausages. Arihara et al. (1998) showed that the potentially probiotic strain *Lact. gasseri* JCM1131 can be used for meat fermentation to enhance product safety, while Sameshima et al. (1998) demonstrated the advantage of potential probiotics *Lact. rhamnosus* FERM P-15120 and *Lact. paracasei* subsp. *paracasei* FERM P-15121 in meat fermentation. Also Erkkila et al. (2001b) reported that *Lact. rhamnosus* strains, especially strains GG and E-97800, are suitable for use as probiotic starter cultures in fermented dry sausages. When not added as starter, lactobacilli may occur in raw meat as a result of fecal contamination. *Lact. plantarum* and *Lact. paracasei* are the most common lactobacilli from oral and rectal mucosae of healthy human subjects (Ahrnè et al., 1998). This would explain the abundant recovery of *Lact. paracasei* in this research. This species is not often found in meat products but, as result of the selective procedure of isolation used in this study, non-dominant strains of *Lact. paracasei* could be isolated which might have been lost if a traditional isolation method had been used. Despite their rare occurrence in meat, Jahreis et al. (2002) used *Lact. paracasei* strains as starters in sausage fermentation. Moreover, both *Lact. plantarum* and *Lact. paracasei* have been found in the human intestine and recognised as probiotics (Ouwehand et al., 2002; Johansson et al., 1993).

Bile plays a fundamental role in specific (Marteau et al., 1997) and non-specific (Kalambaheti, Cooper, & Jackson, 1994) defence mechanism of the gut and the magnitude of its inhibitory effect is determined primarily by the bile salts concentration (Charteris, Kelly, Morelli, & Collins, 2000). In the human gastrointestinal tract, the mean bile concentration is believed to be 0.3% w/v and it is considered as critical and high enough to screen for resistant strains (Gilliland et al., 1984). This concentration was chosen in this study to evaluate the growth capability of the 28 selected *Lactobacillus* strains in the presence of bile salts. All the isolates were able to grow in the medium containing 0.3% bile salts with different levels of resistance. Chateau, Deschamps, and Hadj Sassi (1994) reported that there was extreme variability of resistance to bile salts in the *Lactobacillus* isolates and all the strains tested showed a delayed growth when compared to a reference culture without bile salts. This delay was found in other studies on several *Lact. acidophilus* strains, when the isolates were inoculated in a medium containing 0.3% bile salts (Gilliland et al., 1984; Gupta et al., 1996; Mustapha, Jiang, & Savaiano, 1997). Our results perfectly fit with the above findings: all the 28 *Lactobacillus* strains tested, showed a delay of growth in MRS broth supplemented with 0.3% bile salts. Moreover, there was considerable variability in resistance among the different species of *Lactobacillus* and among the strains belonging to the same species, supporting the importance of considering the tolerance of microorganisms to bile as a selection step for potential probiotics. In our study four strains were particularly resistant to bile salts, three of which belonged to the *Lact. plantarum*-group, and one to the species *Lact. brevis*. The most sensitive strain was

identified as *Lact. reuteri*. When the growth capability in the presence of bile salts, after pre-exposure in PBS at pH 2.5 was investigated, it was possible to distinguish only one strain unable to grow, belonging to the *Lb curvatus-sakei* group. All the other 27 isolates were able to grow in the medium containing 0.3% bile salts. The progress of growth observed by measuring the OD_{600} reached after 28 h of incubation at 37 °C allowed us to recognize the strain CL6 of *Lact. plantarum*-group as the most resistant, while four strains (two of *Lact. casei*-group, one *Lact. brevis* and one *Lact. reuteri*) as the most sensitive. Moreover, by comparing the bile resistance of each strain with and without pre-exposure in PBS at pH 2.5 after 24 h, it was possible to note a growth decrease in 16 strains as a consequence of the inhibitory effect of the low pH. Particularly, the strain GL1 of *Lact. plantarum*-group displayed the same growth in bile salts either with or without pre-exposure. Interestingly, the remaining 11 strains displayed increased growth after pre-exposure demonstrating a high tolerance not only at low pH, but also in presence of bile salts. Prasad et al. (1996) in a similar experiment investigated the ability of three strains of *Lactobacillus* and one strain of *Bifidobacterium* to grow in presence of bile salts, after pre-exposure to low pH (3.0). They found that these strains not only survived the low pH/high bile conditions, but also showed normal growth at bile concentrations up to 1%.

5. Conclusion

We selected at least 20 *Lactobacillus* strains capable of surviving the pH of the stomach and the environment of the intestine, which make them potential probiotics. Of course, these strains do require further in vitro and in vivo investigations such as adhesion to cultured human intestinal epithelial cells, antagonistic activity against enteropathogenic microorganisms, bacteriocins production and antibiotic resistance. Moreover, after specific studies on their technological properties in meat fermentation, the suitable strains may be eventually used as probiotic starter cultures in novel dry fermented sausages manufacture.

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