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Full Length Research Paper

Polyphasic identification of a new thermotolerant species of lactic acid bacteria isolated from chicken faeces

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Two thermotolerant and desiccation tolerant lactic acid bacteria (TDLAB) were pointed out from twenty isolated strains from soils and dried chicken faeces. Samples were collected in poultry farms in the vicinity of Dakar, Senegal (West Africa). The two new isolates were called *Sp.*4 (*Sp.*4=CWBI-B534=LMG7278) and *Sp.*20 (*Sp.*20=CWBI-B545=LMG7279). They are Gram-positive, catalase-negative, facultatively anaerobic, non-motile, and non-spore-forming rods. Both produce D/L lactic acid via homofermentative pathway. Growth of the strains occurred between 15°C and 44°C. The optimum temperature for growth was in 30°C-37°C temperature and pH 3-8 range. Desiccation treatment in glycerol showed 30% survival rates. Complex total fatty acid pattern of the strains showed the presence of $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$. SDS-PAGE of total protein of both strains placed them in *L. plantarum* group. AFLP analysis showed a phylogenetic proximity of the two strains with *L. plantarum stricto sensu* species. Specific amplified 16s rDNA restriction analysis (ARDRA) of the 16S rDNA gene, however, showed that these thermotolerant strains were not *L. plantarum*. ITS sequencing revealed that *Sp.*20 (LMG 7279) could be classed into *Lactobacillus paraplantarum* species since the short sequence of ITS showed 95% of similarity with reference species. Polyphasic identification shows that *Sp.4, (*the type strain is LMG 7278^T) represent a new species within the genus *Lactobacillus* with only 88%+/-1 ITS sequence similarity with reference species. For which the name *Lactobacillus aminata sp. nov.* is proposed.

Key words: *Lactobacillus*, thermotolerant and desiccation tolerant lactic acid bacteria, intergenic transcribed sequence, 16s rDNA restriction analysis, AFLP, SDS-PAGE.

INTRODUCTION

Lactic acid bacteria (LAB) comprise a diverse group of Gram-positive, non-spore forming bacteria and widely involved in the production of fermented foods (Kandler and Weiss, 1986). They are responsible for lactic fermentation in several products such as dairy products, ensilage, ham and sausage. They also play the obviously important role in human and animal health with regard to their use as probiotics (Herrero et al., 1996, Tannock et al., 1999). Among promising probiotic species are members of *genera Lactobacillus, Bifidobacterium and Enterococcus* (Indu Pal Kaur et al., 2002).

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In addition to the requirements for good safety and functional properties, LAB and particularly probiotic formulations should be able to withstand food processing and storage conditions (Svensson *et al*., 1999). A big challenge associated with LAB production is therefore the loss of viability during processing (Desmond, *et al*., 2002). Two inactivation mechanisms are known for LAB: thermal and dehydration inactivation (Lievense *et al*., 1994b). Thermal inactivation is caused by denaturation of critical cell components, mainly DNA and RNA. Cytoplasmic membrane damage is generally considered as the main mechanism of dehydration inactivation (Lievense and van'Riet, 1994a). Despite the improvement that occur the culture, the low survival rate during desiccation remains a major problem (Hodzic *et al*., 1995).

LAB belong to the genus *Lactobacillus* have been isolated from a variety of habitats, including plant, dairy products, meat products, sewage, manure, humans and animals (Kandler and Weiss, 1986). Some new *Lactobacillus sp*. have been isolated from chicken faeces and intestine, including: L. *aviarius* from intestine of chicken (Fujisawa *et al*., 1984), *L. gallinarum* and *L. Johnsonii* from chicken faeces (Fujisawa *et al*., 1991), *L .fermentum subsp.cellobiosus* and *L. animalis* from gastro-intestinal tracts of chicken (Gusils *et al*., 1999). Most of these strains so far have been mesophiles. Some LAB used in diary industry, for example *L. delbrueckii, L. helveticus and S. thermophilus* are already known as thermotolerant starter species from their temperature range of growth (Delcour, *et al*., 2000). We still have little knowledge about the biodiversity of thermotolerant lactic acid bacteria (TLAB) in nature, because very few studies have been done that focus on LAB from the standpoint of their thermotolerance (Niamsup *et al*., 2003). The isolation of *Lactobacillus sp*. from chicken faeces under high temperatures has been reported for the first time by Niamsup *et al*., (2003). But the isolation of *Lactobacillus sp*. from chicken faeces under simultaneously high temperature and dehydrated conditions (very low water activity) has not been reported. The screening of microorganisms from drastic environments appears, as a good strategy to obtain useful strains with simultaneously thermotolerance and high desiccation tolerance. There may have been a strong evolutionary pressure that has favoured some mechanisms increasing the tolerance to desiccation (Pichereau *et al*., 2000). However, to decipher desiccation tolerance mechanism, the topic is not the resistance of particular known structures like spores and akinetes, but rather the tolerance to extreme desiccation of vegetative cells like many *Cyanobacteria*. Understanding the mechanism of desiccation tolerance holds promise not only because it may resolve a cell biology enigma, but also, because, it may find biotechnological applications.

The achievement of the stabilization of dried cells will make possible the production and the storage of an increasing variety of bacterial strains in manufacture of food or pharmaceutical preparations. In this present study, two new isolates selected on the basis of heat and desiccation tolerance is described.

MATERIALS AND METHOD

Samples and cultivation

Soils and faeces samples were collected from different poultry farms in Dakar (Senegal, West Africa). Strains isolation was carried out according to Nakayama et al. (1967). Five grams of sample were mixed with 100 ml of GYP medium contained 1% glucose (w/v), 1% yeast and 1% peptone. The sample suspension was heated for 10 min at 80°C and incubated anaerobically at 30°C. After 48 h of incubation, 100 µl of the mixture were spread onto the surface of GYP agar containing 1% $CaCO_3$. The culture was incubated anaerobically at 30°C. Acid producing bacteria were recognised by the clear zones around the colonies. Bacteria were purified by several isolations and fresh cultures of these isolates were conserved at –80°C with glycerol (30%) as cryoprotective agents.

Phenotypic characterisation

Cells morphology was studied with a light microscope by phase-contrast (Zeiss Axiocam, x 100) and Gram determination was performed. Catalase production was determined by transferring fresh colonies from MRS (De Man et al., 1960) agar to a slide glass and adding H_2O_2 (30%). Pseudo-catalase production was determined by benzidine test according to Gerhardt et al. (1981). Growth conditions were studied in MRS broth from 15°C to 55°C, and in 3 to 8 pH range values. Lactic acid configuration was determinated by enzymatic method, according to manufacter's instructions (Boehringer Mannheim). Carbohydrate fermentation patterns were determined using API 50 CHL test (Bio Mérieux) in duplicate at 30°C.

Thermotolerance of each isolate was estimated after heating bacterial suspension at 80, 90 and 100°C for 10, 10 and 5 min, respectively. Viability assays were performed after dilution on GYP agar (containing 1% of $CaCO_3$) count. The plates were incubated anaerobically at 30°C. Time of decimal reduction at 80°C and 90°C (D_T) was calculated by following equation:

$$D_T = - t/[\text{Log } (N/No)]$$

t: time (minutes), N: survival cell count, No: initial cell count.

Thermoresistant isolates were subjected to a hyperosmotic stress to determine their tolerance to desiccation. The bacterial cells were suspended in glycerol solution with Aw (water activity) = 0.17 for 7 days (Sonan, 1997). Viability was estimated epifluorescence microscope AxioCam HRc type (Zeiss) with FITC excitation filter according to Boulos et al. (1999) modified method with two fluorescent dead/live markers: rhodamine 123 and the propydium iodide. *Rhodococcus erythropolis* T902 (Weekers et al., 1997) and *L. plantarum* G100-CWBI-B076 were used as references. Pictures were analysed using Axiovision (Zeiss) software package.

SDS-PAGE

Cultures were grown anaerobically on MRS agar for 24 h at 28°C. The preparation of the cell extracts and the gel electrophoresis (SDS-PAGE) were carried out as earlier described (Pot et al., 1994). The normalised and digitised protein patterns were numerically analysed and clustered with the reference profiles (culture collection strains and industrial isolates) in the 'LAB' database as currently available (GelCompar™ 4.2 software, Applied Maths, Belgium).

FAME analysis

Total lipids extraction was performed according to the adapted method of Ito et al., (1969). Fatty acid methyl esters were prepared by incubating the lipid extracts at 80°C for 4 h in 2 ml of CH_3OH, containing 15% (v/v) of KOH. The fatty acids were extracted with chloroform and analysed by gas chromatography on a HP 6890 (Hewlett Packard, Waldbronn, Allemagne) equipped with a flame-ionization detector and a SP™–2560, 100 m*0.25 mm*0,2 µm fused silica capillary column. The conditions were as follows: injector temperature, 260°C; detector temperature, 260°C; carrier gas (helium) flow rate, 3 ml/min. The oven temperature was programmed from 140°C during 5 min to 240°C at 4°C/min. For peak identification, standard solution (Sigma) was used.
The results were relative percentages of fatty acids, determined from peak areas of methyl esters. They were means of three independent experiments. The ratio between the standard deviations and the means values was between 2 and 5%.

Amplification of ITS and 16S rDNA

For DNA preparation, cells were cultivated in MRS broth at 30°C. They were harvested in the late exponential phase and DNA was extracted essentially as described by Marmur (1961).
Universal primers were used to amplify the 16S rDNA and intergenic transcribed sequence (ITS) genes. For ITS amplification, forward R16: 5'-GGGTGAAGTCGTAACAAGGTA-3' and reverse R23: 5'-KASTGCCAGGGCATCCAGCGT-3' were used. These primers are derived from conserved regions of 16S and 23S rDNA genes, respectively, and can be used to amplify the ITS sequence of all prokaryotic DNA tested so far. For 16S rDNA amplification, forward1 (PO): 5'-GAAGAGTTTGATCCTGGCTCAG-3' and reverse 1 (P6): 5'-CTACGGCTACC TTGTTACGA-3' were used.
The oligonucleotides were purchased from Eurogentec (Liège, Belgium). Each PCR mixture (50 µl) contained a reaction mix of 20mM Tris-HCL, 20mM of each deoxynucleoside triphosphate, 0.1 mM of each primer, 1 U of the *Taq* polymerase (Eppendorf) and 50 ng of DNA template. All amplifications were carried out in a Master Cycler Personal (Eppendorf) and the following programme was used: initial denaturation for 5 min at 95°C, 30 cycles of denaturation (1 min at 95°C), annealing (1 min at 55°C), and extension (2 min at 72°C). The amplified products were separated in a 1.5% (w/v) agarose gel electrophoresis in TAE buffer, at 120 mA followed by ethidium bromide staining. Gels were photographed with Polaroid Type 667 positive film using a 260 nm UV source.

AFLP

Total genomic DNA is digested by two restriction enzymes (*Eco*RI and *Taq*I). Small DNA molecules (15-20 bp) containing one compatible end are ligated to the appropriate 'stricky end' of the restriction fragments. Both adaptors are restriction halfsite-specific, and have different sequences. These adaptors serve as binding sites for PCR primers.

For this study, adaptor A (*Eco*RI, hexacutter):

```
5′-CTCGTAGACTGCGTACC-3′
      3′-CTGACGCATGGTTAA-5′
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and adaptor B (*Taq*I, tetracutter):

```
5′-GACGATGAGTCCTGAC-3′
    3′-TACTCAGGACTGGC-5′
```

were used.

Some restriction fragments were amplified selectively: PCR primers are specifically hybridised with the adaptor ends of the restriction fragments. Since the primers contain at their 3'-end one or more so-called 'selective bases' that extend beyond the restriction site into the fragment, only those restriction fragments that have the appropriate complementary sequence adjacent to the restriction site will be amplified. Following primer combination was used:

```
E01: 5′-GACTGCGTACCAATTCA-3
T01: 5′-CGATGAGTCCTGACCGAA-3′
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PCR products are separated according to their length on a resolution polyacrilamide gel using DNA sequencer (ABI 377). Fragments that contain an adaptor specific for the restriction halfsite created by the 6-bp cutter are visualised due to the 5'-and labelling of the corresponding primer with the fluorescent dye FAM.
The resulting electrophoretic patterns were tracked and normalised using GeneScan3.1 software (Applera, USA). Normalised tables of peaks, containing fragments of 50 to 536 bp, were transferred into the BioNumerics™ 2.5 software (Applied Maths, Belgium). For numerical analysis, data intervals were delineated between the 50- and 500 bp bands of the internal size standard. Clustering of the pattern was done using the Dice coefficient and the UPGMA algorithm. The profiles were compared with the reference profiles of the lactic acid bacteria taxa as currently available in LMG database (Gent, Belgium).

Amplified 16S rDNA Restriction Analysis (ARDRA)

Aliquots of 10 µl of 16S rDNA PCR products were digested in a 20 µl final volume with restriction endonucleases as specified by the manufacturer. The following enzymes were used: *AccII, Taq I* (Pharmacia). According to Ingrassia et al. (2001), LAB 16S rDNA can be characterised by digestion with *Taq I* and *AccII*.
Restricted DNA fragments were analysed by horizontal electrophoreses in 2 % agarose gel. Gels were stained and photographed as described previously.

16S rDNA and ITS sequencing

The amplified 16S rDNA was purified by using the Microcon YM-100 (Millipore) system according to the manufacturer's instructions. Each amplified ITS was purified after cutting corresponding fragment from agarose gel using Qiaex II gel extraction system (Qiagen). After a second amplification, ITS fragment was purified a second way using the Microcon YM-100 (Millipore).
The determination of nucleotide sequences was performed by Sanger (1977) method using an applied Biosystems 3100 DNA sequencer, the protocols of the manufacturer (Applied Biosystems, USA) and the BigDye ABI® Prism® terminator v3.1 cycle

Table 1. Oligonucleotides used as primers for 16S rDNA sequencing.

Primer	Position	Length (bp)	Sequence
F1 (forward)	21-39	18	5'CTGGCTCAGGAYGAACG-3'
R1(reverse)	533-551	18	5'CTGCTGGCACGTAGTTAG-3'
F2 (forward)	374-392	18	5'GAGGCAGCAGTMGGGAAT-3'
R2 (reverse)	778-796	18	5'AATCCTGTTYGCTMCCCA-3'
F3 (forward)	742-759	17	5'ACACCAMTGGCGAAGGC-3'
R3 (reverse)	1100-1118	18	5'CCAACATCTCACGACACG-3'
F4 (forward)	961-979	18	5'GCACAAGCGGYGGAGCAT -3'
R4 (reverse)	1241-1262	21	5'TGTGTAGCCCRGGTCRTAAG-3'

M=G, A; Y=T, G, C; R=A, T: on *L. plantarum*

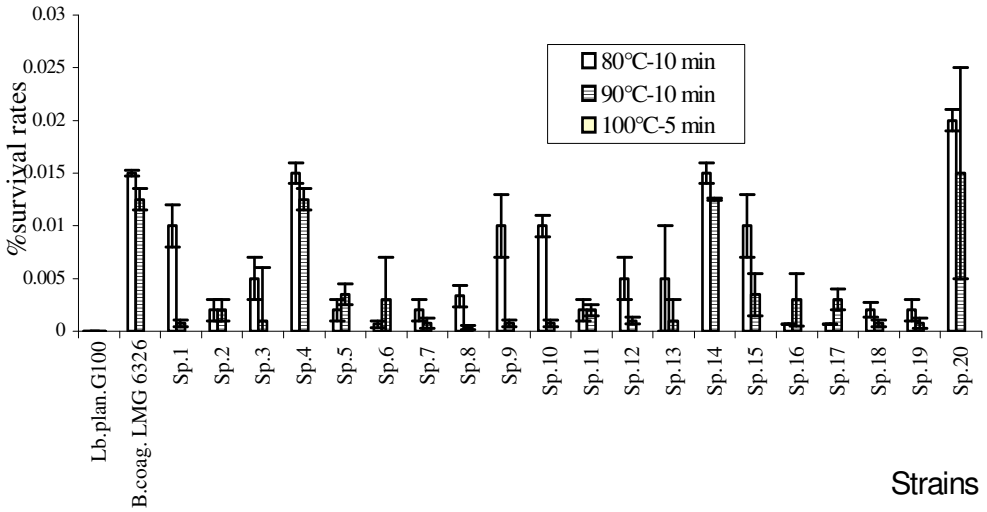

Figure 1. Representative survival rates of TLAB (*Sp.*) and control strains after heat treatment at 80°C, and 90°C for 10 min. No resistance was observed when cells were heated at 100°C for 5 min.

sequencing ready reaction kit. The sequencing primers used for ITS sequencing are the same used for PCR amplification; those for 16S rDNA are listed in Table 1. They were designed by Oligoplayer software, on the basis of comparison of the 16S rRNA gene sequences of several species of *Lactobacilli*. These sequences come from the data banks of the EMBL and were available via service SRS of BEN (Belgian EMBnet-http://www.embnet.org). The position indicates the annealing of the primer 3' end to *E. coli* 16S rDNA in forward (F) or reverse (R) orientation. The electrophoretic profiles were analysed by Genescan Analysis software (Applied Biosystems). Forward and reversed sequences were aligned and compared using the Revseq and Clustal software available on BEN (http://www.be.embnet.org) site.

Sequence assembly was performed by using Vector NTI software. The sequence were compared to sequences available in Genbank database by using FASTA 3 (http://www.ebi.ac.uk/fasta33/) and the results obtained were used to identify isolates to genus or species level. Phylogenetic trees based on the neighbour-joining method were constructed by using Systat 3.0 software. The 16S rDNA nucleotide sequences obtained were aligned within the most similar ones of the Ribosomal Database Project.

Nucleotide sequence accession numbers

Bacterial ITS and 16S rDNA sequences obtained in this study were deposited in Genbank nucleotide database and are available under following accessions numbers (Genbank via Bankit: http://www.ncbi.nlm.nih.gov/BanKIt).

Sp4=CWBI-B534= LMG 7278, 16S rDNA complete sequence: AY594275

Sp4=CWBI-B534= LMG 7278, short ITS complete sequence: AY594277;

Sp20=CWBI-B545= LMG 7279, 16S rDNA complete sequence: AY594276;

Sp20=CWBI-B545= LMG 7279, short ITS complete sequence: AY594278.

RESULTS

Isolation of thermotolerant lactic acid bacteria

Twenty thermotolerant lactic acid bacteria (TLAB) were isolated from soils and chicken faeces samples, by heating them at 80°C for 10 min. All of them are Gram-positive rods, catalase and pseudo-catalase-negative, homofermentative, and non-spore-forming. These new isolates are called *Sp.*1 to *Sp.*20.

The results of thermotolerance (survival rates at various temperature values, Figure 1) showed that after

Table 2. Dt values determination for the best heat resistant strains: Sp.4, Sp.14, Sp.20 and *B. coagulans* LMG 6326.

	$D_{80°C}$ (min)	$D_{90°C}$ (min)
Sp.4	2.62	2.50
Sp.14	2.62	2.56
Sp.20	2.70	2.62
B. coagulans LMG 6326	2.64	2.63

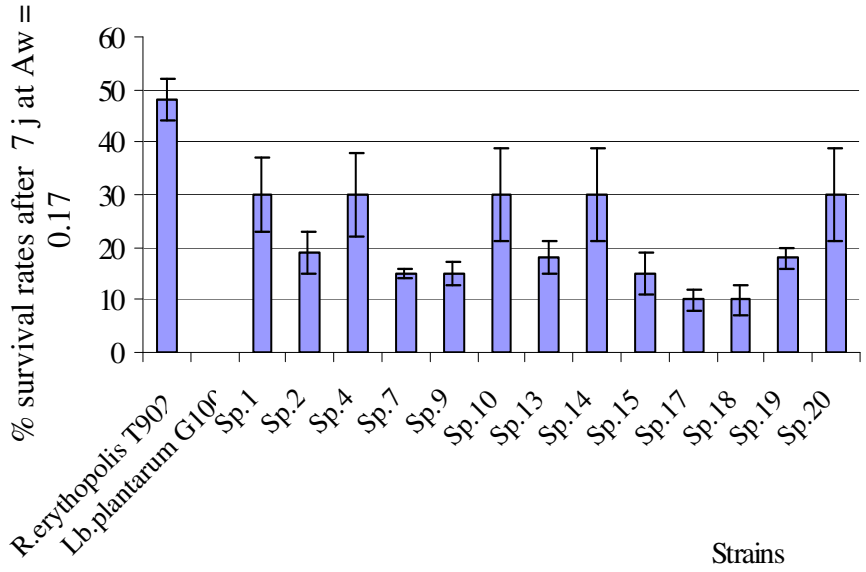

Figure 2. Residual viability (bacterial suspension had a week in glycerol solution, Aw =0.17). Cells were treated with fluorescent markers (rhodamine 123 and propidium iodide using modified method of Boulos et al., 1999). *Sp.3, 5, 6, 8, 11, 12 and 16* did not survived after osmotic treatment like *L. plantarum G100, a sensitive strain.*

treatment, survival cell counts of *Sp.4, Sp.14, Sp.20* and *B. coagulans* LMG6326 (a control strain) were higher.

There was no survival for any strains after heating at 100°C for 5 min. D_T value is the time recurred at fixed temperature to reduce 90% of lived cells. For vegetative forms, generally, $D_{70°C}$ are between 10-15 s and for spore forming bacteria, $D_{84°C}$ are between 2 min. Table 2 shows that $D_{80°C}$ and $D_{90°C}$ values for vegetative heat tolerant cells of *Sp.*4, *Sp.14* and *Sp.*20 were in the same values range as *B. coagulans* LMG6326, a spore-forming strain.

Determination of TLAB desiccation tolerance

Cells were maintained at low water activity (Aw=0.17) in all glycerol solution. After hyperosmotic treatment (for 7 days) at Aw = 0.17, the loss of viability was different from one strain to another (Figure 2). This reference strain (*Rhodococcus erythropolis* T902) has been previously described by Weekers et al. (1997) to have good dehydration resistance.

In reference to *R. erythopolis* T902, the control stain, only *Sp.1, Sp.4, Sp.10, Sp.14 and Sp.20* show better resistance with 30% of viability after desiccation treatment. The variation of water activity in the environment of the bacterial cells has a significant effect on the cellular viability (Lievense *et al*., 1994b).

Physiological analysis

Physiological patterns of selected strains were compared to two control strains; *L. plantarum* G100-CWBI B76 and *B. coagulans* LMG 6326. All strains produce D/L lactic acid from glucose (Table 3). The optimum temperature of growth for all strains is between 30°C and 37°C except *Sp.*4 and *Sp.*20, where growth occurred between 15 to 44°C. The control strains were inactive at 15°C. The pH

Table 3. Physiological characteristics (lactic acid production, pH and growth temperature) of the *Sp.*4, *Sp.*20, *L. plantarum* G100 and *B. coagulans* LMG 6326.

Strains	Acid lactic	Growth Temp (°C)					Growth pH						
		15	30	37	44	55	2	3	4	5	6	7	8
Sp.4	D/L	+	++	++	++	-	-	+	+	++	++	++	++
Sp.20	D/L	+	++	++	++	-	-	+	+	++	++	++	++
L. plantarum G100	D/L	-	++	++	-	-	-	-	++	++	++	++	++
B. coagulans LMG 6326	D/L	-	++	++	+	-	-	-	-	++	++	++	++

Table 4. Total fatty acid composition and modifications induced by growth temperature.

Fatty acids	*L. plantarum G100*		*L. tolerans LMG 9191*		Sp.4			Sp.20		
	30°C	37°C	30°C	37°C	30°C	37°C	42°C	30°C	37°C	42°C
$C_{14:0}$	4.8±0.9	4.2±1.2	0.8± 0.6	3.3± 0.9	3.5± 0.9	4.2±1. 5	2.0± 0. 9	2 0±0.9	2.1±0.6	2.7±0.9
$C_{16:0}$	22.2±2.2	30.3±2.1	7.3±1.8	24.3± 2.2	22.5±2.1	30.4±2.2	16.2±2.3	16.7±2.5	25.1±2.6	24.1±2.5
$C_{16:1}$	4.7 ±0.8	4.1±1.5	1.7±0.6	5.0±0.9	4.3±0.8	2.6 ±0.9	2.4±0.9	3.5±0.9	3.0 ±0.9	1.8± 0.9
$C_{18:0}$	2.6±0.6	8.3±1.5	1.6±0.6	7.6±1.0	2.60±.9	11.4±1.6	6.2±1.3	1.7±0.9	4.9±1 ;8	6.8±1.7
$C_{18:1}$	29.1±2.1	27.8±2.2	53.5±2.2	36.8±2.0	37.0±2.0	25.5±2.2	46.7±3.1	35.8±2.3	25.7±2.5	44±3.2
$C_{18:2}$	0.0	8.2±1.8	0.0	5.6±.9	0.0	3.5 ±0.9	2.2±0.9	0.0	1.6±0.8	2.9± 0.9
$C_{18:3}$	14.2±1.5	8.5±1.3	10.5±1.4	10.5±1.6	10.5±1.2	13.0±1.8	17.3±2.0	23.4±1.9	31.2±2.7	10.7±1.5
SFA	29.6	42.8	9.7	35.2	28.6	46.0	24.4	20.4	32.1	33.6
MUFA	33.8	31.9	55.2	41.8	41.3	28.1	49.1	39.3	28.7	45.8
PUFA	14.2	16.7	10.5	16.1	10.5	16.5	19.5	23.4	32.8	13.6
MUFA+ PUFA	48.0	48.6	65.7	57.9	51.8	44.6	68.6	62.7	61.5	59.4
(MUFA+ PUFA)/SFA	1.6	1.2	7.0	1.6	2.0	1.0	3.0	3.0	2.0	2.0

SFA: saturated fatty acids, MUFA: mono-unsaturated fatty acids, PUFA: poly-unsaturated fatty acids.

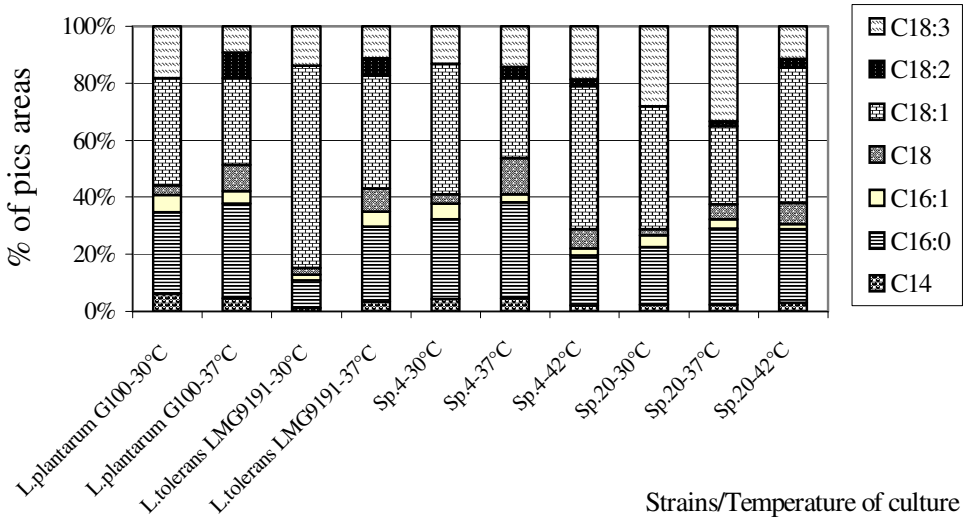

Figure 3. Total cellular fatty acid composition analysis in SP.4, SP.20 and control strains, in Reletion with growth temperature.

at which growth occurs for *Sp*.4 and *Sp*.20 was found in pH 3-8 range values. *L plantarum* G100 did not growth under pH 4 and *B. coagulans* LMG 6326 did not growth under pH 5. The pattern of fermented carbohydrates on API 50 CHL galleries showed a diversity of fermentative metabolism in comparisonb with the control strain and with two thermotolerant strains (G12 and G22) isolated by Niamsup et al. (2003). All strains produce lactic acid from glucose, ribose, L-arabinose, D-fructose, melibiose, D-raffinose and from gluconate. G12 and G22 ferment D-xylose, but not galactose and lactose, while *Sp*.4 and *Sp*.20 ferment galactose and lactose but not D-xylose.

Fatty acid analysis

Study fatty acid modification in relation with temperature is a suitable method to discriminate TLAB strains (Niamsup et al., 2003). The changes in lipid composition enable the microorganisms to maintain membrane functions in the face of environmental fluctuations. In particular, the temperature-induced variations in lipid composition of bacteria are generally thought to be associated with the regulation of liquid-crystalline-to gel-phase transition temperature for the maintenance of an ideal 'functional' physiological state of cell membrane. The fatty acid composition analysis, in relation with growth temperature is given in Tables 3 and 4.

Thermotolerant strains, including *L. tolerans* LMG 9191*, Sp.4 and Sp.20,* have ~20% of unsaturated fatty acids (mono + poly) more then *L .plantarum* G100. *L. plantarum* G100 and *L. tolerans* LMG 9191 are not be able to grow at 42°C. In order to maintain membrane fluidity when temperature of culture increase from 30°C to 37°C (normal LAB growth temperature), all strains reduced synthesis of unsaturated fatty acid (C18:1, C18:3) and increase synthesis of saturated fatty acid (C16:0, C18:0-Table 4-figure 3). Such behaviour was already observed by Russell et al. (1984, 1989, 1990, 1999, 2002) and Grahay et al. (1987).

The oleic acid rate increases when temperature increase from 37°C to 42°C, out of normal LAB growth temperature range (Table 4). Such modifications seemed to be common to eucaryotes and procaryotes. They were already notified by Steel et al. (1994) on *S. cerevisiae* during heat ant oxidative stresses. The increase of the proportion of oleic and linoleic acids with temperature has also been observed in the thermotolerant *Hansenula polymorpha* (Wijeyaratne et al., 1986). In this yeast species, the relative percentage of UFA proved to be higher at 50°C than at 20°C. Guerzoni et al. (2001) also reported the same modifications in *L. helveticus* in the presence of multiple stress factors, such as low pH and high NaCl concentration. These authors show that oleic acid level increased when *L. helveticus* was submitted to the combinations of acid, osmotic and heat stress.

SDS-PAGE of total proteins

The dendrogram of the cluster analysis with a subset of the reference strains (culture collection strains and industrial isolates from the BCCM/LMG LAB database, Gent, Belgium, BCCM.LMG@RUG.ac.be) is given below (Figure 4). The type strains are indicated by ^{T}. *Sp*.4 (7278) and *Sp*.20 (7279) are in a cluster within *plantarum* group SDS-PAGE cannot separated them from the three species which constituted this group. The protein patterns in Figure 4 show three clusters: *L. pentosus* cluster, *L. plantarum/L. paraplantarum* cluster and *L. alimentarius/L. farciminis* cluster. *Sp*.4 (7278) and *Sp*.20 (7279) seemed closely related to *L. pentosus*.

16S rDNA sequences comparison

The 16S rDNA sequences of *Sp*.4 and *Sp*.20 were determined and compared with available 16S rDNA sequences in the Genbank database. The sequences were similar to each other (>99% similarity). This approach is not suitable because of high identity value shared by *L. plantarum* and *L. pentosus* (Collins et al., 1991; Quere et al., 1997).

AFLP results

The *plantarum* group has a genetic heterogeneity, which was demonstrated by Dellaglio et al. (1975), on the basis of DNA/DNA hybridisation data. Three groups have been identified which were later classified as *L. plantarum stricto sensu*, *L. pentosus* (Zanoni et al., 1987) and *L. paraplantarum* (Curk et al., 1996). These three species are closely related genotypically and show highly similar phenotypes. So, a correct identification of some new isolates as one of these strains is complicated by the ambiguous response of traditional physiological tests and molecular methods (Torriani et al., 2001). The dendrogram of the AFLP clusters analysis with reference strains (*L. plantarum*, *L. pentosus* and *L. paraplantarum,* Figure 5) shows that *Sp*.4 and *Sp*.20 constituted separated subgroups within *plantarum* species, in contrast with SDS-PAGE results.

PCR-ARDRA of 16S rDNA

16S rDNA was amplified from three *L. plantarum* strains and from *Sp.4* and *Sp*.20. Fragments of 1500 bp were obtained. The Figure 6 presents the patterns of the 16S rDNA polymorphism after *Acc II* restriction digestion. All *L. plantarum* patterns (Figure 6, lanes 2, 3 and 4) after digestion with *AccII*, show the same restriction profile characterised by the presence of three fragments (~175, 300 and 450 bp). *Sp*.4 and S*p*.20 pattern (lanes 5

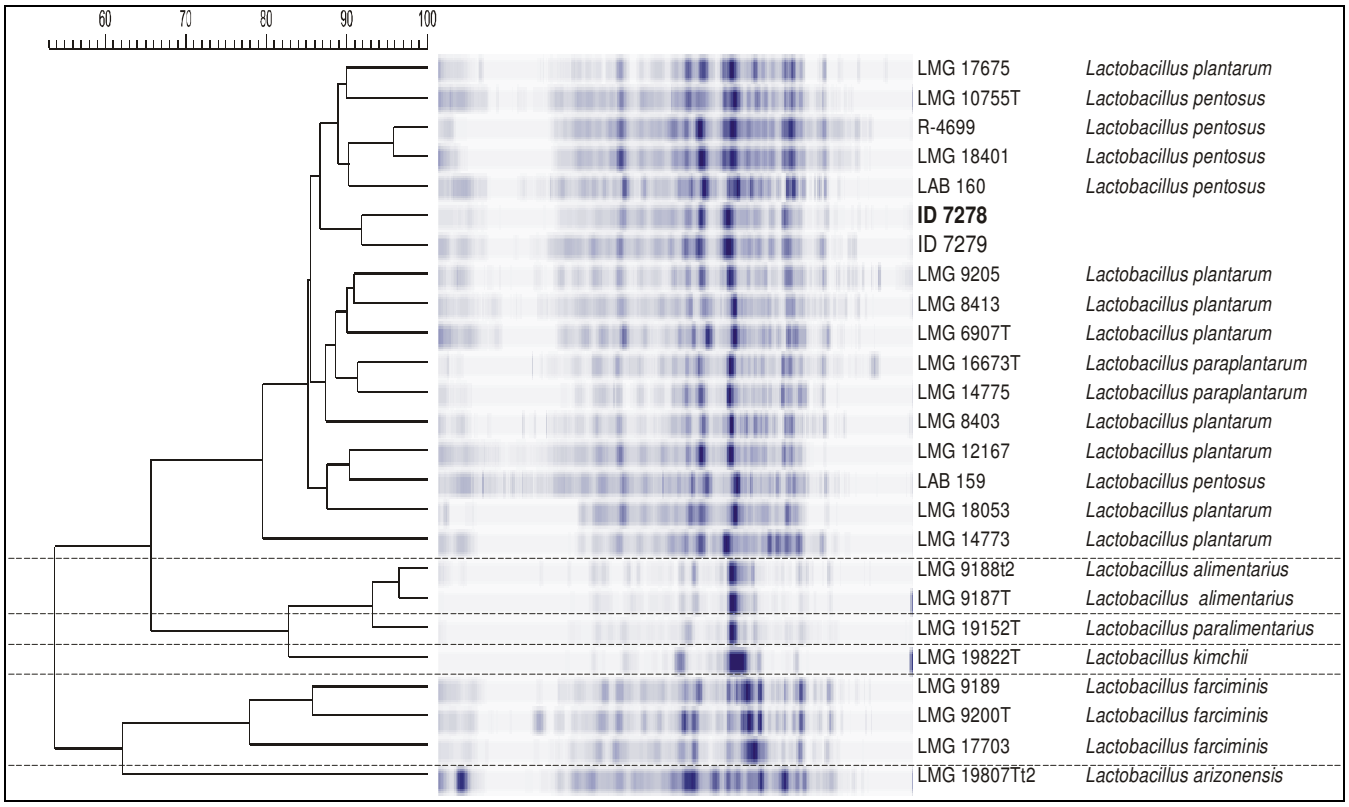

Figure 4. Dendrogram showing the relationship between electrophoresis protein patterns of *Sp.4* (7278), *Sp.20* (7279) and LMG LAB database strains.

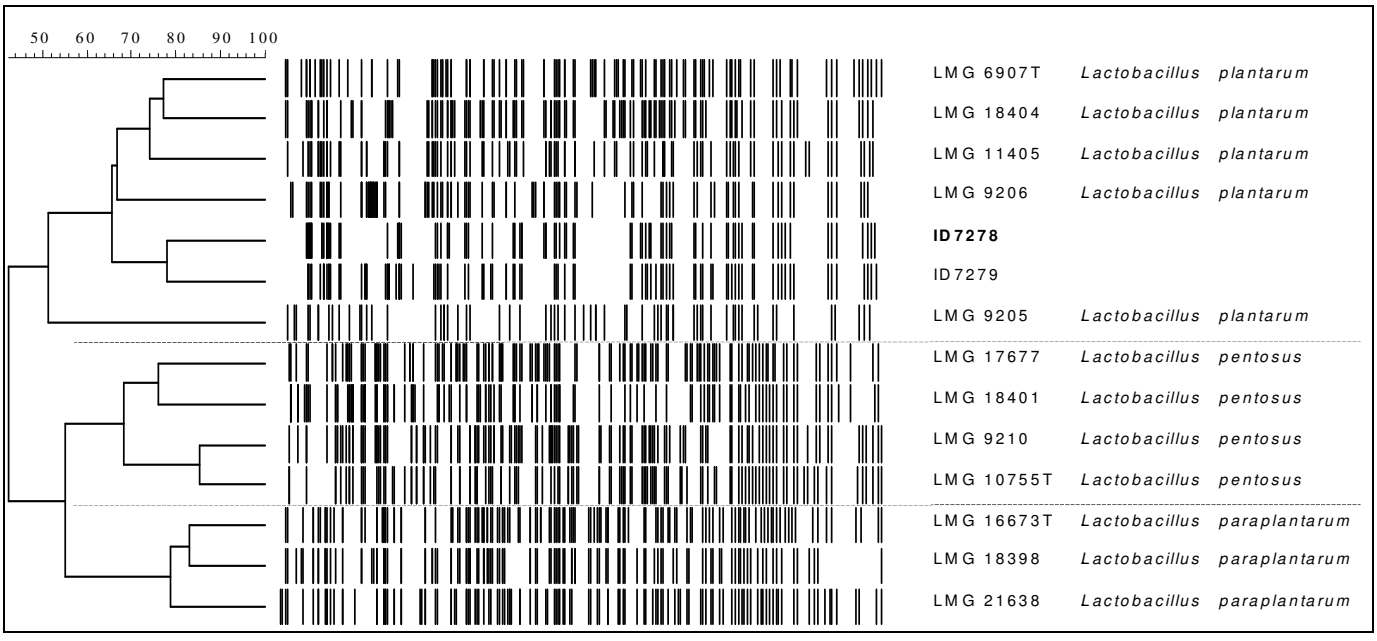

Figure 5. Dendrogram showing the relationship between AFLP profiles of *Sp.4* (7278), *Sp.20* (7279) and reference strains (LMG LAB database).

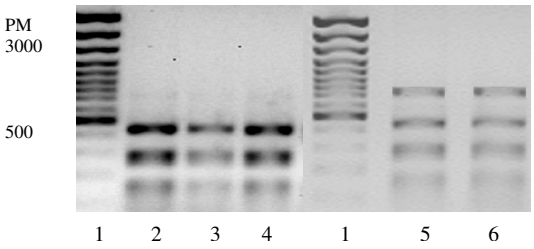

Figure 6. Photo-negative ethidium bromide stained agarose gel electrophoresis of amplified 16S rDNA of strains digested with *AccII*. 1: 100 bp DNA ladder used as molecular size marker (PM); 2: *L. plantarum G100*; 3: *L. plantarum L115;* 4: *L .plantarum 'Ma'*; 5: *Sp*.4; 6: *Sp*.20.

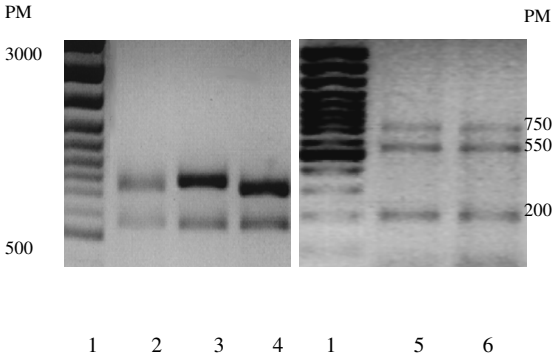

Figure 7. Photo-negative ethidium-bromide stained agarose gel electrophoresis of amplified 16SrDNA of strains digested with *Taq I.* Lane 1: 100 bp DNA ladder used as molecular size marker (PM); Lane 2: *L. plantarum G100*; Lane 3: *L. plantarum L115*; Lane 4: *L. plantarum 'Ma'*; Lane 5: *Sp*.4; Lane 6: *Sp*.20.

Table 5. Restriction fragment length polymorphism obtained after 16S rDNA digestion with *Acc II* and *Taq I*. Sizes were expressed in bp.

Strains	**Restriction enzymes**	
***L.plantarum* reference**	*AccII*	*TaqI*
L. plantarum G100	450-300-175	750-550
L. plantarum "Ma"	450-300-175	750-550
L. plantarum L115	450-300-175	750-550
New TDLAB		
Sp.4	700-450-300	750-550-200
Sp.20	700-450-300	750-550-200

and 6)) show a new pattern with ~300, 450 and 700 bp fragment. According to this result, it may be affirmed that *Sp*.4 and *Sp*.20 are not *L. plantarum.*

All *L. plantarum* patterns (lanes 2, 3 and 4) show after digestion with *TaqI* the same restriction profile with two fragments (~550, 750bp). *Sp*.4 and S*p*.20 both show (figure 7) a different profile from the one of *L. plantarum,* with three fragments (~200, 550 and 750 bp). These results, confirm that *Sp*.4 and *Sp*.20 are genetically different from *L .plantarum.* Restriction fingerprinting profiles are given in Table 5 with different fragment sizes.

Table 6. Homology values calculated (Vector NTI, Maryland, USA) for short ITS sequences.

	Homology (%)					
	G100	**Sp.4**	**Sp. 20**	**DSM 10667**	**U97138**	**U97134**
G100	100	86	94	92	91	92
Sp.4	86	100	95	89	88	87
Sp.20	94	95	100	96	95	89
DSM 10667	*92*	*89*	*96*	*100*	*95*	*92*
U97138	91	88	95	95	100	92
U97134	92	87	89	92	92	100

G100=*L. plantarum G100-CWBIB076,*
DSM10667=*L. paraplantarum* DSM10667
U97138=*L. paraplantarum* U97138,
U97134=*L. pentosus* U97134.

Intergenic transcribed spacer (short ITS) sequencing and sequence alignment results

ITS of *Sp*.4, *Sp*20 and *L .plantarum* G100 were sequenced and these sequences were compared with those of *L. plantarum stricto sensu, L. pentosus and L. paraplantarum,* found in NCBI Genbank. Sequences alignment did not introduce any gaps, and there were no ambiguous positions that could lead to bias in the inference of phylogeny. This was not surprising, since the sequences were derived from closely related species. Table 6 shows homology percentages calculated for different ITS sequences (Vector NTI)
Comparing short ITS homology (Table 6), *Sp.20* could be classified into *L. paraplantarum cluster* with 95% of ITS similarity with *L. paraplantarum* U97138 and 96% of ITS similarity with *L. paraplantarum* DSM10667. Moreover, the percentage of homology between reference species and thermotolerant strains are way out the permissive value (97.5%), proposed by Tannock et al. (1999) to classify two strains in the same species. Therefore, *Sp.4* isolate could not be classified into any existing taxon. *Sp.4* represent a new species (Figure 8) within *Lactobacillus* genus with only 88%+/-1 ITS sequence similarity with reference species. Figure 8 shows phylogenetic relationship between *L. plantarum*, *L. paraplantarum, L. pentosus, Sp.4* and *Sp. 20* based on short ITS alignment.

Sp. 20 and Sp.4 as inferred by the neighbour-joining method with short ITS sequence alignment. *Sequences were obtained from NCBI Genbank. Alignments were realised on Vector NTI Software.

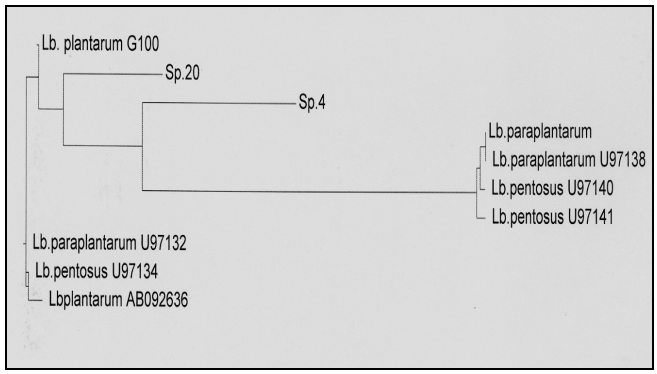

Figure 8. Phylogenetic tree showing the relative positions of *L. plantarum*, *L. paraplantarum*, L. pentosus*,*

DISCUSSION

Twenty strains were isolated from soils and dried chicken faeces. Two strains (*Sp*.4 and *Sp*.20) are resistant to both heat and desiccation stresses. These strains produce massive amount of lactic acid; they are catalase negative, Gram positive and non-spore-forming rods. A polyphasic approach was employed for the taxonomic identification of these two strains, including techniques with a wide discriminative power (growth conditions, auxanography, fatty acids analysis, SDS-PAGE of proteins, genomic AFLP, 16S rDNA gene restriction analysis, and ITS sequencing). Culture studies showed large range of growth temperatures (15°C to 44°C) with an optimum at 30-37°C and pH values (3 to 8). Sugar fermentation profiles obtained by API 50 CHL indicated that *Sp*.4 and *Sp*.20 have heterogeneous carbon metabolism. They ferment lactose and galactose but not D-xylose, in contrast with thermotolerant strains recently isolated by Niamsup et al. (2003).

The cellular fatty acid composition is the result of a sum of complex phenoma maintaining optimal viability of cell under stress conditions (Russell et al., 1995). Linoleic acid probably results from oleic acid after conversion by desaturase activity (Guillot et al., 2000). It seemed that survival and growth of *Sp.4* and *Sp.20* at 42°C can be related to the high proportion of MUFA and to an overall equilibrium in unsaturated/saturated fatty acid ratio. Some thermotolerant strains, (Niamsup et al., 2003) present similar oleic acid rates when cultures were incubated at 42°C. Guillot et al. (2000) reported that in *Lactococcus lactis*, at high temperature, the UFA/SFA increases from 1.7 to 2.7. An oxygen-dependent desaturase induction was postulated in thermotolerant yeast strains with the roles of preventing increased oxygen and reactive oxygen species (ROS) accumulation in the membrane at superoptimal temperatures and protecting the cells from damage generated by oxidative and thermal stresses (Guerzoni et al., 1997). It seemed that TLAB thermal responses are similar to those reported by Guerzoni et al. (1997).

These results clearly identify an increase in unsaturation level of fatty acids as a response to exposure to superoptimal temperatures. This behaviour suggests that desaturase activation or hyperinduction plays an important role in the response to heat stress at least in thermotolerant strains. In addition, FTIR spectroscopy provided evidence for changes in membrane fluidity as result of the unsaturation of fatty acids (Szalontai et al., 2000). According to these authors, the frequency of symmetric CH_2 stretching is higher in unsaturated fatty acid, because of loading. The decrease of this frequency enhances rigidification of membranes (Szalontai et al., 2000). In *Lactobacilli,* temperature is reported to induce chances in fatty acid in relation to the regulation of the degree of fatty acid unsaturation, cyclization and proportions of long-chain fatty acids containing (Dionisi et al., 1999). However, the range of temperatures generally studied has not exceeded the optimal ones (except in Niamsup et al., 2003). Moreover, fatty acid or precursor supplementation in culture media strongly affects cellular fatty acid profiles. In particular, oleic acid is known to be incorporated into LAB membrane.

SDS-PAGE protein profile analysis indicated that these isolates constitute a homogeneous phenon/subgroup within *plantarum* group, even if they cannot be separate from *L. paraplantarum, L. pentosus and L. plantarum.* It is possible that environmental adaptation (to heat and dehydration) performs suitable physiological modifications and induces typical metabolism profiles. Some of regulatory mechanisms responding to an environmental stress condition are common to those found in Gram-positive bacteria, and many stress responses are conserved at the protein level. It was demonstrated by Prasad et al. (2003) by enhancing protective effect of prestressing *L. rhamnosus* HN 001 with heat or osmotic shock on the viability of dried preparations during storage.

These results confirm the insufficiency of biochemical and physiological analysis to characterise new wild isolates, because the metabolism can be modified with evolution and environment. We further complete this taxonomic study using genotypic techniques with diverse taxonomic discriminative powers (Torriani et al., 2001). AFLP analyses confirm that *Sp*.4 and *Sp*.20 cannot be separated to *plantarum* group, since the three constituted species are phylogenetiquely closely related. The 16S rDNA sequences of *Sp*.4 and *Sp*.20 were determined and compared with available 16S rDNA. sequences in the Genbank database. % of 16S rDNA homology to each other is up to 99% similarity. This approach is not suitable because of high identity value shared by *L. plantarum* and *L. pentosus* (Collins et al., 1991; Dellaglio et al., 1975; Quere et al., 1997). PCR-ARDRA, which has been successfully used for differentiation of a variety of microorganisms (Moschetti et al., 1997; Ventura et al.,

2001) is rather preferred. The 16Sr DNA was amplified and, after restriction with *AccII* and *TaqI,* a polymorphism was found with each restriction enzyme between *L. plantarum* control strains *Sp*.4 and *Sp*.20. ARDRA technique described here can be considered as a reliable and a reproducible molecular tool to discriminate new isolated from *plantarum stricto sensu* species (Garcia-Martinez et al., 1999).

ITS (short fragment) of *L. plantarum* G100, *Sp.4 and Sp.20* were sequenced and compared with those of *L. plantarum stricto sensu, L. paraplantarum and L. pentosus.* Sequence analysis revealed that *Sp*.20 belonged to *para*p*lantarum* species with 95-96 % of ITS homology. *Sp*.4 appears like a new species within the genus *Lactobacillus* with only 88% ITS sequence similarity with reference species and the name *Lactobacillus aminata sp. nov* is proposed for it.

Lactobacillus aminata sp. nov (*Sp*.4) *c*ells are Gram-positive, non-motile, non-spore-forming, catalase-negative rods of 1x2-3 µm in size, which occur singly, in pair or as, short chain. After growth at 30°C for 48 h, colonies on GYP (glucose yeast peptone) agar are ~1-2 mm in diameter, white, convex, circular and smooth, obligatory homofermentative and produce large among of D/L lactic acid. It grows at 15 to 44°C and from pH 3 to 8. It does not hydrolyse aesculin, does not produce hydrogen sulphide, does not reduce nitrate, does not liquefy gelatin and does not produce dextran from sucrose. Acid is produced from glucose, ribose, L-arabinose and D-fructose, melibiose, saccharose, galactose, methyl α-D-mannoside, mannose, mannitol sorbitol, thehalose, maltose, lactose, arbutine, melezitose, D-turanose and D-raffinose. Glycerol, erythritol, D-arabinose, L-xylose, methyl-β-xyloside, dulcitol, inositol, inulin, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate are not fermented. The major cellular fatty acid produced when culture was incubated at 42°C is a straight chain unsaturated acid, C_{18}:$_1$. The GenBank accession number for *Sp.4* 16S rDNA complete sequence is AY594275 and Sp.4 short ITS complete sequence is AY594277*. L. aminata* has been deposited in the BCCM/LMG Bacteria Collection (University of Gent, Gent, Belgium) as LMG ID 7278 and in the Centre Wallon de Biologie Industrielle- Unité de Bio-industries (CWBI) Culture Collection (Faculté de Sciences Agronomiques de Gembloux, Belgium) as CWBI-B534. Isolated from faeces of chickens, coming from Senegal, the type strain is *Sp.4* (=LMG ID 7278^T=CWBI-534).

This study demonstrates the usefulness of polyphasic criteria for identification of natural isolates, which often show wide microbial heterogeneity (Dykes et al., 1994). The ability to discriminate between closely related strains and the capacity to identify unusual or atypical isolates can be improved by combining different techniques, such as physiological analysis, SDS-PAGE of proteins, AFLP, ARDRA and ITS sequencing.

Use of lactic acid starter is more and more important since potential health benefits were showed (Daly et al., 1998). So, freezing is widely used for long-term preservation of their viability, easy transport and technological properties (acidification activity, aroma compounds production, probiotic potentialities). However, freezing causes a loss of viability acidification activity of LAB (Fonseca et al., 2003). Moreover, bacterial resistance to stress including freezing, frozen storage and desiccation depends on the strain and on production conditions, stabilisation (concentration, cryoprotection, freezing) and storage (Fonseca et al., 2001).

The aim of this work was to isolate and identify thermotolerant and desiccation tolerant strains. The next step will to estimate potential properties like probiotic and behaviour of *Sp*.4 and *Sp*.20 during storage at room temperature. Such strains have been prepared by their background to support drastic environment (heat, low Aw). In nature, they are exposed to variable factors, such as temperature, and the availability of nutrients and water. Survival in this changing environment requires a wide range of fast, adaptive responses (Van de Guchte et al., 2002). In the majority of cases, the bacterial response leads to transcriptional activation of genes whose products cope with a given physico-chemical stress. Gene regulators respond to specific signals (such as environmental and cellular signals) by stimulating or inhibiting transcription, translation, or some other event in gene expression, so that the rate of synthesis of gene products is appropriately modified. Microorganisms that are able to offer a successful physiological/biochemical adaptation are better suited to colonising the changing niche (Ramos et al., 2001). In this study, new isolates are able to give suitable response to desiccation and heat stresses. They are be able to support storage at room temperature (data not published), where cytoplasmic membrane damages are generally considered as the main mechanism of dehydration (Lievense et al., 1994b). Economically and practically, it will be a real progress in dairy industry to screen rustic LAB strains particularly in developing hot countries even in all over the world.

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