

Isolation and identification of some *Lactobacillus* and *Enterococcus* strains by a polyphasic taxonomical approach

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Abstract

The studied lactic acid bacteria (strains LRV 1, LRV 2, SP, S 1 and S 2 respectively) are part of a set of 50 strains newly isolated from calf ruminal liquid, pig stomach and silage. They are Gram-positive, catalase negative, facultative anaerobic, non-motile and non-spore forming rods (LRV 1, SP, S 1) or cocci (LRV 2 and S 2). Optimal growth temperature occurred between 28^oC to 42^oC (strain dependent) into a pH range between 3.0 to 8.0 and up to 0.5% NaCl.

The pattern of fermented carbohydrates on API 50 CHL galleries showed a diversity of fermentative metabolism for isolates LRV 1, SP and S 1.

Testing with API 20 STREP showed that LRV 2 fermented raffinose and hydrolyzed hippuric acid while S 2 did not ferment raffinose and hippuric acid.

16S rDNA RFLP analysis confirmed similarity between strains belonging to the same species therefore supporting inclusion of the conspecific strains into the same OTU.

Polyphasic identification showed that LRV 1, SP and S 1 represented three different strains of *Lactobacillus plantarum* subsequently denominated as *Lactobacillus plantarum* CMGB 1, *Lactobacillus plantarum* CMGB 2 and *Lactobacillus plantarum* CMGB 3 while LRV 2 and S 2 were two different strains of *Enterococcus faecium* that were subsequently denominated as *Enterococcus faecium* CMGB 6 and *Enterococcus faecium* CMGB 8.

Keywords: isolation of lactic acid bacteria, physiological tests, 16S rDNA restriction analysis

Introduction

Lactic acid bacteria (LAB) comprise a large and diverse group of Gram positive, non-spore forming bacteria, catalase negative, able to produce lactic acid as the main end-product of the fermentation of carbohydrates.

LAB have been isolated from specific habitats, including dairy products, plants, meat products, sewage, manure humans and animals. Some new LABs have been isolated from chicken faeces and from gastro-intestinal tracts of chicken, calf, pig, etc. (4, 5, 7).

LAB species have been identified on the basis of cell morphology, analysis of various carbohydrate substrates. In general phenotypic methods lack of reproducibility generated by conditions of culture related to different laboratories and to diversity of strain. Selective culture media and phenotypic test enable lactobacilli to be differentiated from morphologically similar bacteria (8). During the last decade molecular methods like RAPD, AFLP, RFLP, ARDRA sequencing, DNA hybridization (2, 8, 12, 13), PFGE, FIGE (1) have been applied extensively for intraspecific and genotyping of new isolates. The accurate identification of *Lactobacillus* and *Enterococcus* species can be stimulated through the

similitude of molecular sequences (16S rRNA) encoding genes and it is based on assumption that rRNA genes are highly conserved, horizontal gene transfer phenomena among organisms have not involved those genes and the amount of similarity of sequences between different individuals (3, 9, 10, 11).

The main goal of our study was the identification of new LAB isolates by polyphasic taxonomy techniques.

Material and Methods

Isolation of lactic acid bacteria was performed from the following typical niches: micro-silos stored silage (after 2 days of fermentation), ruminal liquid collected from calves at six weeks of age (prior to ablactating), and pig stomach.

Cultivation media: lactic acid bacteria were isolated on MRS (6) pH 5.5 or 6.5 while enterococci were isolated on LIA (g/l: lactose-10.0; bactotripton-10.0; yeast extract -5.0; gelatin -2.5, sodium chloride -4.0; ascorbic acid -0.5), pH 7.0.

The isolation method varied according to the source. In the case of ruminal liquid and pig stomach 1 mL of sample were incorporated into liquid MRS and incubated for 24 hours in order to obtain enriched cultures. Serial dilutions were prepared from the enriched cultures and 0.1 mL from each dilution were plated on MRS or LIA and incubated for 24 - 48 hours at 37°C. The 1 g silage sample was thoroughly mixed with 9 mL of isotonic solution, serial dilutions were prepared and from each dilution 0.1 mL were plated on MRS and LIA and incubated for 24 - 48 hours.

Biochemical traits of the microorganisms were determined using API kits (BIOMERIEUX): API 50 CHL for the identification of the *Lactobacillus* strains and API 20 STREP for the identification of *Enterococcus*. The results were integrated using the API Web software.

For **determination of the optimal growth temperature** all strains were cultured at 5°C, 15°C, 28°C, 37°C, 42°C and 60°C and optical density at 600 nm was checked after 24 hours.

Resistance to pH variation was examined on MRS at pH 3.0, 4.0, 5.5, 6.5, 8.5 and 9.0, respectively, again monitoring growth by determination of optical density at 600 nm at 24 hours.

Examination of the influence of various sodium chloride concentrations was conducted on MRS containing 0.5%, 5.0%, 8.0%, 10.0% and 12.0% NaCl with growth monitored *via* determination of optical density at 600 nm after 24 hours of incubation.

Amplified 16S rDNA restriction analysis

16S rDNA was amplified using the following primers: GM3F- 5'-AGAGTTTGATCCTGGCTCAG-3' (position 8-24 in *E. coli* 16S rDNA) and GM4R- 5'-GGTTACCTTGTACGACTT- 3' (position 1492- 1507 in *E. coli* 16S rDNA). 16S rDNA was amplified using a MJ-RESEARCH thermocycler with 1.2 U *Taq* polymerase (PROMEGA)/μg ADN for a total sample volume of 50 μL. A 1.5% agarose check gel was prepared. The amplification products were digested with *Hinf* I and *Hae* III (1.2U/μg DNA). Restriction fragments were migrated into a 2% agarose gel.

Results and Discussion

Physiological tests

Assessment of the ability to use certain sugars

Preliminary identification of LVR 1, SP and S 1 was performed using API 50 CHL with *Lactobacillus plantarum* ATCC 8014 as reference.

Data in Table 1 reveal the existence of differences among the three strains with respect to their capacity of consuming various carbon sources.

LVR 1 and SP exhibited assimilation patterns that were similar to each other but different from that of S 1 and also from the pattern manifested by the ATCC reference. Distinction among the three isolates was based on their ability to use L- arabinose, inositol and α - methyl- D- mannoside as a carbon source. It could also be noticed that the ability to use particular carbon sources is strain- specific, i.e. S 1 can consume 5- keto- gluconate.

It is reasonable to presume that the similarities LRV 1 and SP share in their biochemical profiles are due to their somehow similar origin (from ruminal calf liquid and pig stomach, respectively).

Table 1. API 50 CHL results and preliminary identification of *Lactobacillus plantarum* strains

Substrate	Strains			
	<i>Lb. plantarum</i> ATCC 8014	LRV1	SP	S1
Control	-	-	-	-
Glycerol	-	+	+	+
Erythritol	-	-	-	-
D-arabinose	-	-	-	-
L-arabinose	+	-	-	+
D-ribose	+	+	+	+
D-xylose	-	-	-	-
L-xylose	-	-	-	-
D-adonitol	-	-	-	-
Methyl-BD-xylopyranoside	-	-	-	-
D-galactose	+	+	+	+
D-glucose	+	+	+	+
D-fructose	+	+	+	+
D-mannose	+	+	+	+
L-sorbose	-	-	-	-
L-rhamnose	-	+	+	+
dulcitol	-	-	-	-
inositol	-	+	+	-
D-mannitol	+	+	+	+
D-sorbitol	+	+	+	+
Methyl- α D-mannopyranoside	+	-	-	+
Methyl- α D-glucopyranoside	+	-	-	-
N-acetylglucosamine	+	+	+	+
amygdalin	+	+	+	+
arbutin	+	+	+	+
esculin	+	+	+	+
salicin	+	+	+	+
D-cellobiose	+	+	+	+
D-maltose	+	+	+	+
D-lactose	+	+	+	+
D-melibiose	-	+	+	+
D-saccharose	+	+	+	+
D-trehalose	+	+	+	+
inulin	-	-	-	-
D-melezitose	+	+	+	+
D-raffinose	-	+	+	+
starch	-	-	-	-

glycogen	-	-	-	-
xylitol	-	-	-	-
gentiobiose	+	+	+	+
D-turanose	-	-	-	-
D-lyxose	-	-	-	-
D-tagatose	-	-	-	-
D-fucose	-	-	-	-
L-fucose	-	-	-	-
D-arabitol	-	+	+	+
L-arabitol	-	-	-	-
gluconate	-	+	+	+
2- ketogluconate	-	-	-	-
5-ketogluconate	-	-	-	+
Preliminary identification	<i>Lb. plantarum</i>			
	CMGB 1	CMGB 2	CMGB 3	

The isolates studied were preliminary identified as members of the *Lactobacillus* genus, namely as *Lactobacillus plantarum* (with a 99.9% similarity to the reference) and designated as CMGB 1 (*ex*- LRV 1), CMGB 2 (*ex*- SP), and CMGB 3 (*ex* – S 1), names by which they were registered into the MICROGEN Culture Collection.

LVR2 and S 2 were preliminary identified with the API 20 STREP kit having as reference *E. faecium* IC 14203. The strains differ from one another only with respect to their capacity to hydrolyze hippuric acid and to grow on medium with raffinose (Table 2).

Table 2. API 20 STREP results and preliminary identification of *Enterococcus faecium* strains

Substrate	Strains		
	<i>E. faecium</i> 14203	LRV 2	S2
pyruvate	+	+	+
hippurate	+	+	-
esculin	+	+	+
Pyrrolidonyl 2 naphthylamide	+	+	+
6-bromo-2-naphthyl α D-galactopyranoside	+	+	+
naftol AS-BI β -D-glucuronat	-	-	-
2-naphthyl- β -D-galactopyranoside	+	+	+
2-naftilfosfat	+	+	+
L-leucina-2-naphthylamide	+	+	+
arginine	+	-	-
ribose	+	+	+
L arabinose	+	+	+
mannitol	+	+	+
sorbitol	-	-	-
lactose	+	+	+
trehalose	+	+	+
inulin	-	-	-
raffinose	-	+	-
starch	+	+	+
glycogen	+	-	-
Preliminary identification	<i>E. faecium</i>		
	CMGB 6		CMGB 8

Interpretation of the obtained data resulted into preliminary identification of the two strains as members of the *Enterococcus* genus, species *Enterococcus faecium* (showing 92% similarity to

the reference in the case of LRV 2 and 99.2% in the case of S 2) entering the MICROGEN Culture Collection under the names of CMGB 6 (*ex*- LRV 2) and CMGB 8 (*ex*- S 2).

Determination of growth parameters

Determination of the optimal growth temperature

The optimal growth temperature of the *Lb. plantarum* strains (CMGB 1, CMGB 2, CMGB 3) ranges from 28^o C to 37^o C (see Figure 1). The *E. faecium* strains are able to grow between 28 and 42^o C (Figure 2). Extreme temperatures (i.e. 5^o C or 60^o C) do not allow growth of either of the studied strains.

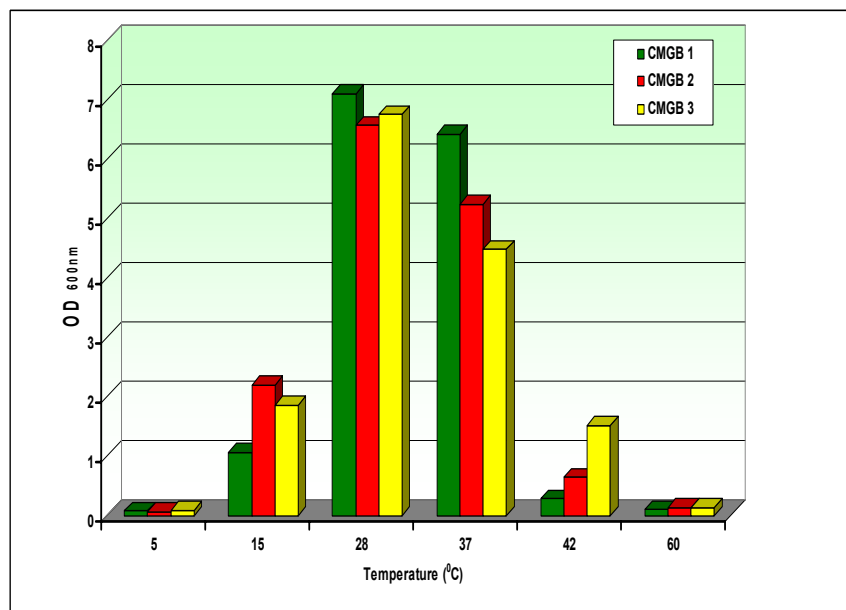


Figure 1. Growth of *Lactobacillus* strains at various temperature values

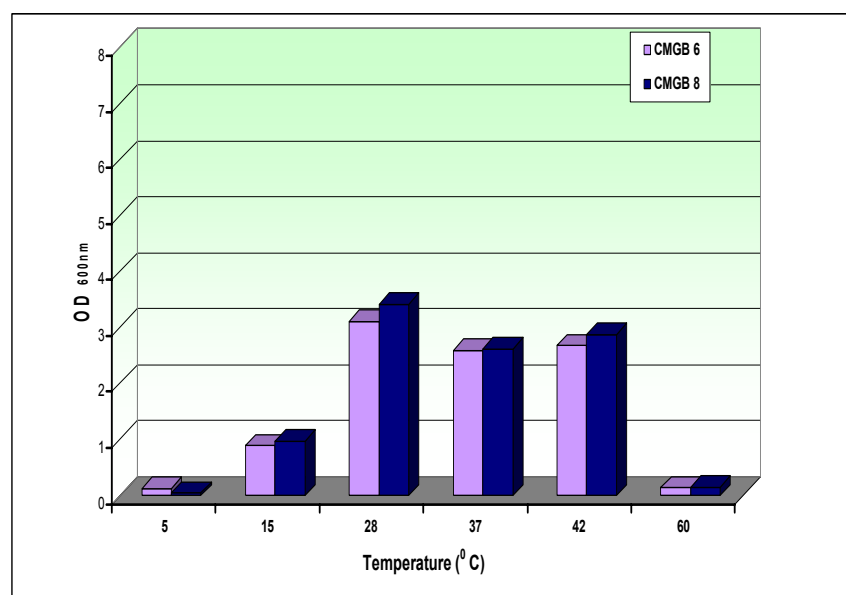


Figure 2. Growth of *Enterococcus* strains at various temperature values

Resistance to pH variation

Development of the strains belonging to genus *Lactobacillus* varies according to the strain (Figure 3). *Lb. plantarum* CMGB 1 exhibits a maximum at 5.5, the intensity of growth decreasing gradually up to a pH value of 8.5. The optimal pH value required for growth of *Lb. plantarum* CMGB 2 ranges from 6.5 to 7.5 (the strain being also able to tolerate pH values as high as 9.5) whilst in the case of *Lb. plantarum* CMGB 3 maximal biomass formation occurs at a higher pH comparing to the previously mentioned strains (namely in the 6.5- 8.5 interval). For *E. faecium* CMGB 6 and CMGB 8 biomass formation is increasing gradually from pH 6.5 attaining a maximum at pH 8.5- 9.5 (Figure 4).

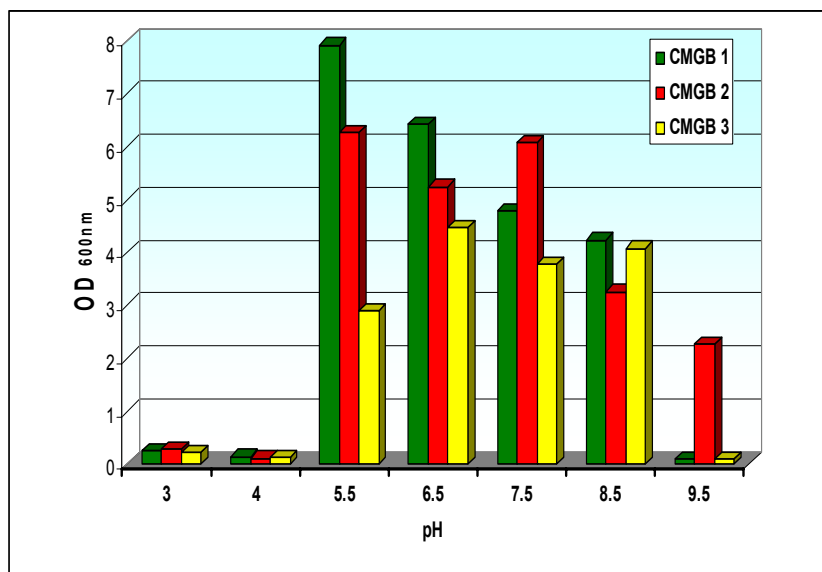


Figure 3. Growth of *Lactobacillus* strains at various pH values

Examination of the influence of various sodium chloride concentrations

Various sodium chloride concentrations in the medium correlate with differences in bacterial growth, namely: growth is optimal at NaCl 0.5% while concentrations of 10-12 % NaCl inhibit biomass accumulation (Figure 5 and 6).

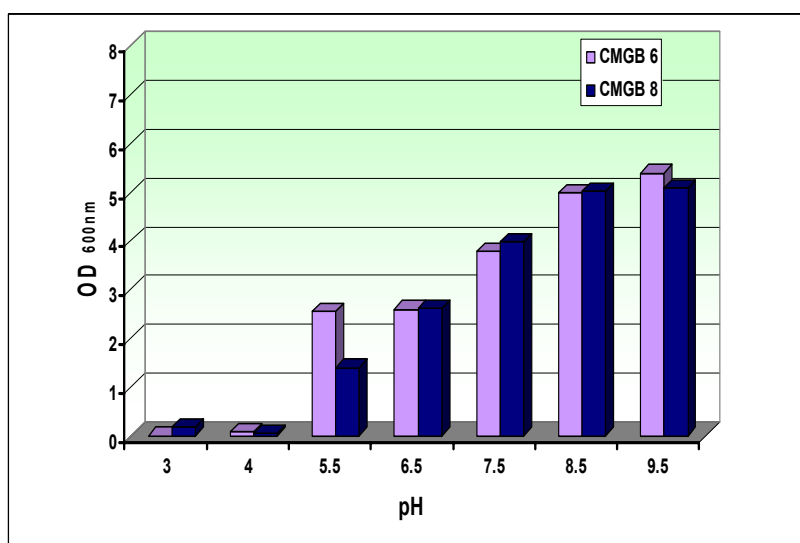


Figure 4. Growth of *Enterococcus* strains at various pH values

Molecular methods of identification

Amplified 16S rDNA restriction analysis

The restriction patterns of the amplified 16S rDNA are shown in Figures 7-10. For the length of the fragments see Table 3.

Results were double-checked by summing up the dimensions of the fragments for each digestion reaction resulting in all cases a value of approx. 1500 bp corresponding to the length of the amplified sequence.

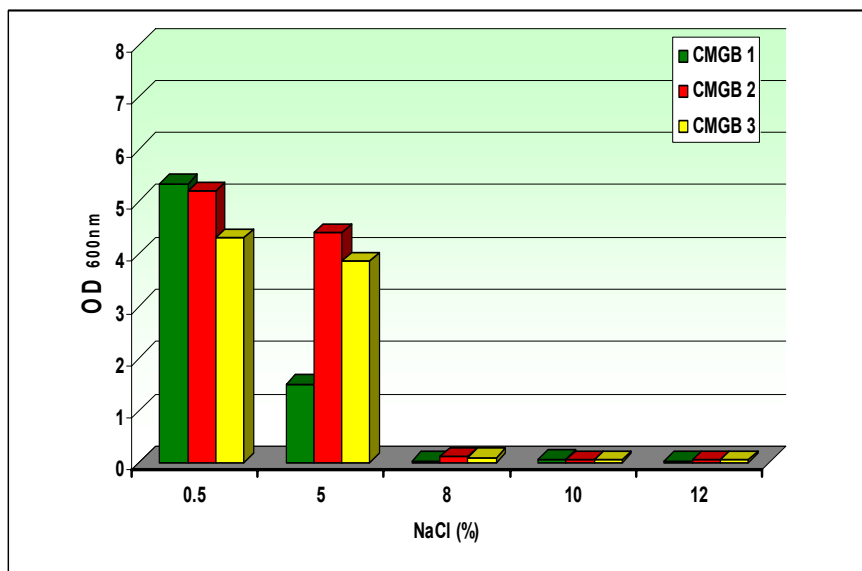


Figure 5. Tolerance of *Lactobacillus* strains to various NaCl concentrations

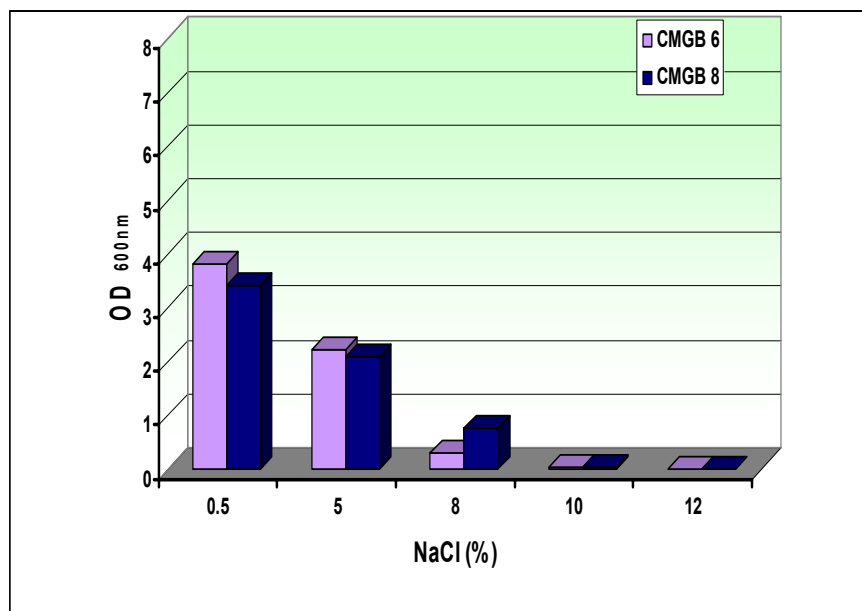


Figure 6. Tolerance of *Enterococcus* strains to various NaCl concentrations

Relying upon the ARDRA patterns it was concluded that *Lactobacillus plantarum* ATCC 8014 and *Lactobacillus plantarum* CMGB 1, CMGB 2 and CMGB 3 belonged to the same OTU.

Strains *E. faecium* CMGB 6 and CMGB 8 respectively do not belong to the same OTU.

Corroborating the results of the conventional tests with the molecular analysis allowed placing of the five newly isolated strains into genera *Lactobacillus* (species *plantarum*, for CMGB 1, CMGB 2 and CMGB 3) and *Enterococcus* (species *faecium*, for CMGB 6 and CMGB 8).

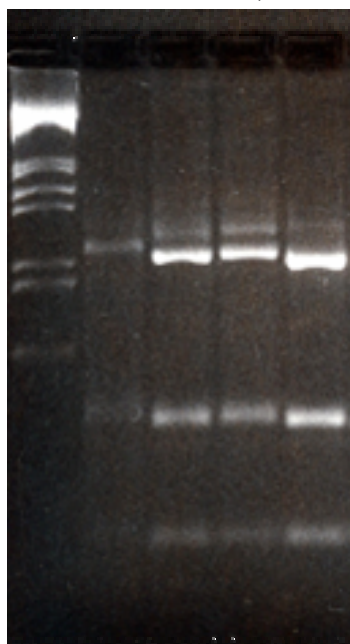


Figure 7. ARDRA patterns (in 2% agarose gel electrophoresis) with *Hinf*I: 1- λ /*Eco*RI+*Hind* III Marker (PROMEGA), 2- *Lb. plantarum* ATCC 8014, 3- *Lb. plantarum* CMGB 1, *Lb. plantarum* CMGB 2, 4- *Lb. plantarum* CMGB 3.

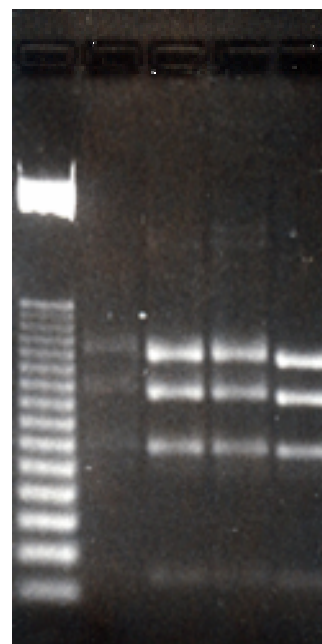


Figure 8. ARDRA patterns (in 2% agarose gel electrophoresis) with *Hae* III: 1- λ /50bp ladder Marker (PROMEGA), 2- *Lb. plantarum* ATCC 8014, 3- *Lb. plantarum* CMGB 1, *Lb. plantarum* CMGB 2, 4- *Lb. plantarum* CMGB 3



Figure 9. ARDRA patterns (in 2% agarose gel electrophoresis) with *Hinf*I: 1 - 100bp DNA ladder (PROMEGA), 2- *E. faecium* 14203; 3- *E. faecium* CMGB 6, 4- *E. faecium* CMGB 8 100bp DNA

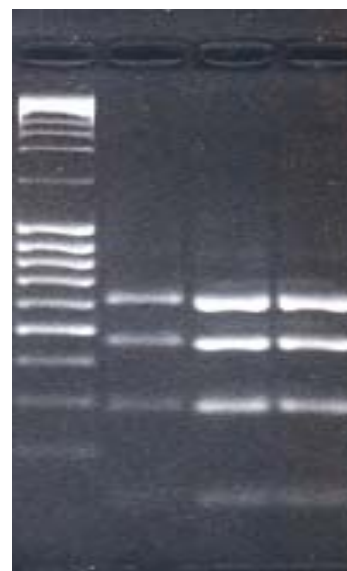


Figure 10. ARDRA patterns (in 2% agarose gel electrophoresis) with *Hae* III: 1 - 100bp DNA step ladder (PROMEGA), 2- *E. faecium* 14203; 3- *E. faecium* CMGB 6, 4- *E. faecium* CMGB 8

Table 3. Dimensions of the restriction fragments as resulting from analysis of the ARDRA patterns

Enzyme	Strain	No. fragments	Dimension [bp]
<i>Hae</i> III	<i>Lb. plantarum</i> ATCC 8014, CMGB 1, CMGB 2, CMGB 3	4	75, 300, 500, 600
	<i>E. faecium</i> IC 14203, CMGB 6, CMGB 8	4	100, 250, 450, 650
<i>Hinf</i> I	<i>Lb. plantarum</i> CMGB 1, CMGB 2, CMGB 3	3	125, 350, 1000
	<i>E. faecium</i> IC 14203, CMGB 6, CMGB 8	3	175, 350, 1000

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