



Original / Otros

Screening in a *Lactobacillus delbrueckii* subsp. *bulgaricus* Collection to select a strain able to survive to the human intestinal tract

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Abstract

Objectives: Genetic diversity and resistance of *Lactobacillus bulgaricus* subsp. *delbrueckii* collection with 100 isolates from different home-made yogurt in rural Bulgarian areas were determined.

Methods: The strain K98 was the most resistant to bile salts and low pH. Survival and effects on short chain fatty acids production were tested in 20 healthy volunteers. High genetic diversity was observed in the *L. bulgaricus* collection by RAPD, whereas the ability of tolerate high deoxycholic acid concentrations, and different acid pHs was variable. The strain K98 was selected and used to prepare a homemade yogurt which was administered to 20 healthy volunteers (500 ml/day during 15d). A basal faecal sample and another after yogurt intake were recovered.

Results: DGGE experiments, using both universal and Lactic Acid Bacteria (LAB) primers, demonstrated no significant changes in the qualitative composition of gut microbiota. A band corresponding to *L. bulgaricus* was observed in all 20 samples. Viable *L. bulgaricus* K98 strain was only recovered in one volunteer. After yogurt intake we found an increase of LAB and *Clostridium perfringens*, and a decrease of *Bacteroides-Prevotella-Porphyromonas*. In addition, increases of acetic, butyric and 2-hydroxy-butyric acids in faeces were detected.

Conclusions: Genetic diversity of *L. delbrueckii* subsp. *bulgaricus* especie is high. We have isolated a probiotic resistant strain to bile and high acidity, *L. delbrueckii* subsp. *bulgaricus*-K98. Qualitative and quantitative changes in the intestinal microbiota are found after ingestion of a homemade yogurt containing this strain, with a concomitant increase in faecal SCFA. Our findings support the interest in developing further studies providing different amounts of *L. delbrueckii* subsp. *bulgaricus*-K98, and should evaluate its clinical effects in human disease.

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Key words: *Lactobacillus delbrueckii* subsp. *bulgaricus*. Antibiotic susceptibility. Bacterial survival. Gut microbiota. Short chain fatty acids.

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ESTUDIO DE UNA COLECCIÓN DE *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS* Y SELECCIÓN DE UNA CEPA CAPAZ DE SOBREVIVIR EN EL TRACTO DIGESTIVO HUMANO

Resumen

Objetivos: Se determinaron la diversidad genética y la resistencia de una colección de más de 100 cepas de *Lactobacillus bulgaricus* subespecie *delbrueckii*, aisladas de diferentes yogures caseros de las áreas rurales de Bulgaria.

Métodos: La cepa K98 fue la más resistente a las sales biliares y al pH bajo. La supervivencia y los efectos sobre la producción de ácidos grasos de cadena corta se evaluó en 20 voluntarios sanos. Se observó una alta diversidad genética en la colección de *L. bulgaricus* mediante RAPD, mientras que la capacidad de tolerar concentraciones altas del ácido desoxicólico y de diferentes niveles de pH fue variable. Se seleccionó la cepa K98 y se usó para preparar un yogur casero que se administró a los 20 voluntarios (500 ml/día durante 15 días). Se recogieron muestras fecales basales y tras la ingesta del yogur.

Resultados: Los experimentos DGGE, empleando cebadores universales y para bacterias ácido-lácticas (BAL) demostraron que no hubo cambios significativos en la composición cualitativa de la composición de la microflora intestinal. Se observó una banda correspondiente a *L. bulgaricus* en las 20 muestras. Sólo se recuperó una cepa viable de *L. bulgaricus* K98 en un único voluntario. Tras la ingesta de yogur, hallamos un aumento de BAL y de *Clostridium perfringens* y una disminución de *Bacteroides-Prevotella-Porphyromonas*. Además, se detectó un aumento en las heces de los ácidos acético, butírico y 2-hidroxibutírico.

Conclusiones: La diversidad genética de *L. delbrueckii* subespecie *bulgaricus* es alta. Hemos aislado una cepa probiótica resistente a la bilis y a la acidez elevada, *L. delbrueckii* subsp. *bulgaricus*-K98. Se hallaron cambios cualitativos y cuantitativos en la microflora intestinal tras la ingesta de yogur casero que contenía esta cepa, con un aumento concomitante en las heces de AGCC. Nuestros hallazgos apoyan el interés por desarrollar estudios futuros con cantidades variables de *L. delbrueckii* subsp. *bulgaricus*-K98, y que evalúen sus efectos clínicos en la enfermedad humana.

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Palabras clave: *Lactobacillus delbrueckii* subsp. *bulgaricus*. Susceptibilidad antibiótica. Supervivencia bacteriana. Microflora intestinal. Ácidos grasos de cadena corta.

Introduction

Intestinal microbiota has gained a great interest owing to its positive effects on the host organism, such as the formation of intestinal wall, resistance to colonization, production of short chain fatty acids (SCFA), group B and K vitamins, and interaction with the mucosal immune system.^{1,2} Human microbiota has been estimated in more than 10¹³ microorganisms of different bacterial species, most of them strictly anaerobic.³ Nevertheless, 99% of the total microbiota is constituted of only 30-40 different bacterial genera, which might vary in their relative proportions.^{4,5} Although a given individual host harbours a particular array of gut bacteria which remains relatively constant over time,⁶ it is well known that several factors modifying normal intestinal microecology, such as diet changes, sex or age, may induce both quantitative and qualitative changes in the composition of intestinal microbiota.^{7,8}

Probiotics have been defined as microorganisms which, once ingested in adequate amounts, have beneficial effects on the host.⁹ Among others, probiotics have been associated with the prevention of antibiotic-associated diarrhoea, as well as rotavirus and *Clostridium difficile* infections in children and adults respectively, reduction of irritable bowel syndrome symptoms, remission of ulcerative colitis and pouchitis, and prevention of necrotizing enterocolitis.² Furthermore, in recent years, probiotics have been shown to have a beneficial effect on obesity and insulin resistance.¹⁰ Despite the current lack of enough evidence, it has been already shown in several clinical trials and basic research that the different beneficial effects of probiotics are strain⁹ and dose-dependent, so the study of a single strain is becoming interesting.^{11,12}

Yogurt has traditionally been considered as a probiotic-carrier food, capable of inducing changes in gut microbiota and, as a result, promoting several health benefits.^{13,14} However, several authors have found contradictory results in the real ability of yogurt bacteria to survive and proliferate in the human intestine.^{15,16,17} These differences can be attributed to several factors: the methods used in bacterial detection, the type of the human population studied, the yogurt manufacturing process, and the volume and duration of its administration. We have recently shown that consumption of either fresh or heated-treated yogurt containing *Lactobacillus delbrueckii* subsp. *bulgaricus* (from now expressed as *L. bulgaricus*) and *Streptococcus thermophilus* produced similar changes in human microbiota, suggesting a predominant prebiotic effect, instead of a probiotic one of yogurt.¹⁸ In fact non-viable *L. bulgaricus* exert effects in mucosal immune stimulation.^{19,20} However, *L. bulgaricus* probiotic properties could be strain-specific,⁹ and its differences in genetic diversity and resistance to relevant environmental conditions for survival in the upper gut have not been explored yet. The *Lactobacillus* genera colonizes the gastric, duodenal and upper jejunum

spaces, which relates with resistance to high acidity and high bile salts concentration, as it occurs with other organisms as *Campylobacter*.^{21,22} The viability of a weak acid-resistant or weak bile-resistant organism will certainly be severely limited, and consequently its ability to reach lower spaces in the gut.²³

Different molecular techniques using total microbiota DNA (microbiome analysis) have been developed to detect and identify microorganisms from complex microbial ecosystems without necessity of being cultured. Genes encoding the 16S-rRNA are excellent phylogenetic markers for genus and species level determination and their sequences have rendered vast information about the composition of human intestinal microbiota, a particularly complex bacterial system. The denaturing gradient gel electrophoresis (DGGE) technique combines PCR amplification of the 16S-rRNA genes with a subsequent electrophoresis of the PCR product in vertical gels in a denaturant gradient, allowing visualization of even 1% of dynamic changes in bacterial communities.²⁴⁻²⁹ Complementary to qualitative changes explored by the DGGE method, the quantitative real-time PCR technique offers the possibility of analyzing the quantitative changes within a particular bacterial group.^{27,30-32}

The aim of this work was to explore the genetic diversity in a large collection of natural *L. bulgaricus* isolates with the aforementioned techniques, and to screen such collection for key-traits allowing tolerance to chemical environmental conditions of relevance for intestinal survival, such as low pH and high bile salts, and absence of mechanisms of antibiotic resistance. This was done in order to select the most environmentally resistant strains, to investigate their ability to survive in the human gut, and to explore their effects on gut microbiota and SCFA generation.

Material and methods

Bacterial isolates and genetic diversity

A total of 100 *L. bulgaricus* isolates collected in homemade yogurts from different rural areas of Bulgaria were included (one strain per yogurt sample). The specie was confirmed by positive amplification with specific primers¹⁶ and comparing the 16S rRNA nucleotide sequences. Isolates were grown on MRS agar (Oxoid, Basingstoke, UK) at 37° C with 10% CO₂ and humidity, and were conserved in semi-skimmed milk at -80° C. Genomic DNA was extracted from single colonies onto MRS agar and a previously published RAPD-PCR scheme was carried out³³ using the primer Lbd 1254 (5'CCGCAGCCAA3'). The resulting electrophoretic patterns were compared with the Phoretix 5.0 software (Nonlinear Dynamics Ltd, UK), and clustering was performed by the unweighted pair group method with arithmetical average (UPGMA), in order to establish the genetic diversity.

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) for penicillin, ampicillin, vancomycin, teicoplanin, erythromycin, gentamicin, kanamycin, streptomycin, fusidic acid, linezolid, tetracycline, moxifloxacin, chloramphenicol, and quinupristin/dalfopristin were determined by microdilution using a cation-adjusted MRS medium (CLSI, 2000). Susceptibility panels were incubated overnight at 37° C in ambient air after inoculation. *Streptococcus pneumoniae* ATCC 49619 and *Staphylococcus aureus* ATCC 29213 were used as controls in each run. Available MIC breakpoints, recommended by the FEEDAP (<http://www.efsa.europa.eu/en/science/feedap.html>), were considered for all antibiotics. For fusidic acid, non-susceptibility MIC value was estimated at 4 mcg/ml.

Tolerance to deoxycholic acid and pH

A 1/10 dilution from each overnight culture in MRS broth was made and then inoculated on a 96-well microtiter plate containing MRS broth (Oxoid, Basingstoke, UK) with different deoxycholic acid (Sigma) concentrations (10, 20 and 40 g/L), or adjusted at different pH concentrations (1, 2, 3, 4 and 5). Positive growth controls without deoxycholic acid or pH variation were included in each run. Plates were incubated in atmospheric conditions at 37° C during 24 hours.

Detection of yogurt bacteria in healthy volunteers

A total of 20 young healthy volunteers (5 males, 15 females, all of them co-workers in our laboratory) with a mean age of 30 ± 5 years were included. Daily-prepared homemade yogurt was made using pasteurized cow's milk, with final bacterial counts of 1.3 x 10⁷ and 2 x 10⁸ CFU/g of the selected *L. bulgaricus* K98 strain and *S. thermophilus*, respectively. Volunteers ingested 500 g of yogurt daily during two consecutive weeks, without imposition of a special diet. Two different faecal samples were recovered per individual: a first baseline sample after a week without yogurt in their diet and a second sample after the homemade yogurt period intake. Faecal samples were processed immediately upon reception as follows: 0.5 g of fresh faeces were suspended in 5 ml of saline and centrifuged at low speed for 5 min (1.000 g). 1 ml of the upper phase was collected; and finally, 100 µl from these samples were consecutively diluted in saline and seeded in MRS agar. After 48 h of incubation, at least five different colonies per morphology were re-seeded and tested by positive amplifications for *L. delbrueckii* specific primers, and finally identified by 16S rRNA nucleotide sequence¹⁶ Identity of all colonies with positive amplifications for *Lactobacillus* genera were

compared with the original isolate *L. bulgaricus* K98 by RAPD analysis.

Denaturing gradient gel electrophoresis (DGGE)

Microbiome DNA extractions were performed in 200 µl of the faecal samples with the commercial system FastDNA Spin Kit for soil (Bio 101 Inc., La Jolla, Calif), and the Fast prep Kit was used for the mechanical break. Finally, 100 ng of DNA were used for each 16S-rRNA PCR reaction. Positive 16S-rRNA amplicons were separated in vertical electrophoresis polyacrylamide gels (8%) at 60° C and the urea-formamide denaturing gel gradient (33-43%) was submitted to 130 V during 330 min. Gels were visualized with ethidium bromide. For *Lactobacillus*-DGGE experiments, the control included a mixture of DNA from *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus reuteri* ATCC 23272, *Lactobacillus brevis* ATCC 14869, *Lactobacillus casei* ATCC 393, and *L. bulgaricus*. DNA from *Bifidobacterium bifidum* ATCC 29521 and *Bifidobacterium infantis* ATCC 15697 were added to those controls used for *Lactobacillus*-DGGE experiments. Primers and optimized PCR conditions used in this study are summarized in table I.

Real time polymerase chain reaction (RT-PCR)

We realised different group-specific assays for determination of the density of Acid Lactic Bacteria, the *Bacteroides-Prevotella-Porphyromonas* group, the *Clostridium coccooides* group, the *Clostridium perfringens* group, and species-specific assays for *Bacteroides vulgatus*. Calibration curves were performed in each experiment with DNA obtained from fresh broth cultures of representative strains: *L. bulgaricus* for the Acid Lactic Bacteria group, *Bacteroides vulgatus* for the Bacteroides group and *B. vulgatus*, *C. perfringens* for the *C. perfringens* group, and finally *Clostridium sphenoides* for the *C. coccooides* group.

Quantitative PCR experiments were performed with a LightCycler 1.5 apparatus, using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Indianapolis, IN, USA). All PCR tests were carried out in duplicate in a final volume of 20 µl containing 50 ng of each faecal DNA preparation. The thermal cycling conditions used were as follows: an initial DNA denaturation step at 95° C for 5 min, followed by 35 cycles of denaturation at 95° C for 15 s; primer annealing at optimal temperature (see table I) for 20 s; extension at 72° C for 30 s, and an additional incubation step at 80-85° C for 30 s to measure the SYBR Green I fluorescence. Finally, melt curve analysis was performed by slowly cooling the PCRs from 95 to 60° C (0.3° C per cycle); with simultaneous measurement of the SYBR Green signal intensity.

Table I
Primers and conditions used in the DGGE and RT-PCR experiments

Primer sequence (5' → 3')	Size	PCR conditions	Reference	
<i>L. bulgaricus</i>	CCTCATCAACCGGGGCT TGATCCGCTGCTTCATTCA CGCCCGGGTGAAGGTG	678 bp	90° C 20 s 55° C 75 s 72° C 40 s	Lick et al., 2001
RAPD				
Lbd 1254	CCGCAGCCAA		Torriani et al., 1999	
DGGE				
Universal Bacteria	907: CCCCCTCAATTC/CTTTGACTTTT Univ 341GC-F:CGCCCGCGCGCGGGCGGGG CGGGGGCACGGGGGCTACGGGAGGCAGCAG	566 bp	95° C 30 s 58° C 1 min 72° C 1min 20 s	Muyzer et al., 1993
LAB group	LacF: AGCAGTAGGGAATCTTCCA LacGC-R: CGCCCGGGCGCGCCCGGGCGGCCCGG GGCACCGGGGGATTYACCGCTACACATG	340 bp	95° 30 s 62° 1 min 72° 1 min	Walter et al., 2000
RT-PCR				
<i>B. vulgatus</i>	BV-F: ACGATGAATACTCGCTGTTTGC BV-R: CATGTTCTCCGCTTGTGC	133 bp	95° 10 s 66° 5 s 72° 9 s	Fujita et al., 2002
<i>Bacteroides</i> group	B-F: GGTGTCGGCTTAAGTGCCAT B-R: CGGA(C/T)GTAAGGGCCGTGC	140 bp	95° 10 s 66° 5 s 72° 9 s	Rinttila et al., 2004
<i>C. perfringens</i> group	Cp-F: ATGCAAGTCGACCGA(G/T)G Cp-R: TATCGGTATTAATCT(C/T)CCTTT	120 bp	95° 10 s 56° 5 s 72° 9 s	Rinttila et al., 2004
<i>C. coccooides</i> group	Ccoc-F: AAATCACGGTACCTGACTAA Ccoc-R: CTTTGAGTTCATTCTTGCGAA	440 bp	95° 10 s 53° 7 s 72° 20 s	Matsuki et al., 2002
LAB group	Lac-F: AGCAGTAGGGAATCTTCCA Lac-R: ATTYCACCGCTACACATG	327 pb ^a	95° 10 s 57° 5 s 72° 15 s	Walter et al., 2000

Short chain fatty acid (SCFA) determination

Adequate dilutions of formic, 2-ketoglutaric, citric, succinic, fumaric, 2-hydroxy-butyric, propionic, lactic, acetic, valeric, isovaleric and butyric acids, all from Sigma, were used as patterns. 0.5 g of dried faeces were suspended in 5 ml of deionized water and prepared as previously described.³⁴ Capillary zonal electrophoresis was performed on a Beckman System 5500 (P/ACE) equipped with an ultraviolet detector set at 200 nm, an automatic injector, and a 37-cm total length (75- μ m i.d.) polyacrylamide gel-pretreated column cartridge. All experiments were carried out at 25° C. Sample injections were made by pressure for 5 s with an applied reversed voltage of 10 kV for buffer S and 15 kV for buffer A. Calibrators were obtained from Sigma Chemical Co.

Statistical analysis

All RT-PCR reactions were performed in duplicate and the results expressed are the mean of both experi-

ments. The software SPSS version 11.5 was used for statistical data analysis. The ANOVA test was applied to analyze the number of bands obtained with the DGGE experiments, whereas non-parametric tests (Kruskal-Wallis, Friedman, and Wilcoxon Mann-Whitney) were applied for comparison of RT-PCR results.

Results

Genetic diversity of L. bulgaricus strains

A high genetic diversity of the 100 isolates obtained from yogurts at rural Bulgarian areas was detected by RAPD-PCR. Two main genetic groups (A and B) were distinguished differing in 0.5 Dice coefficient, with most of the strains belonging to either of these groups (50% and 37% of the strains). At a higher discrimination coefficient (0.7), the A group subdivided into 3 main genogroups, and the B group into 4 genogroups (fig. 1). Intra-group differences were observed, reducing the possibility of duplicates testing.

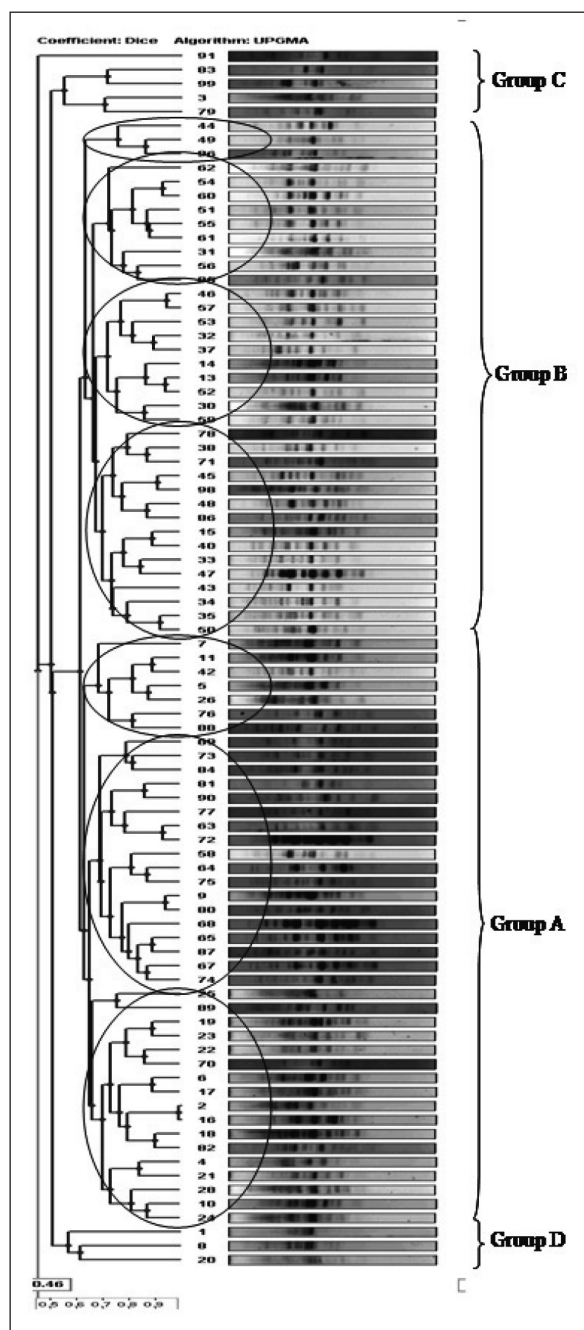


Fig. 1.—Genetic relationship among 100 *L. delbrueckii* subsp. *bulgaricus* isolates by RAPD typing.

Resistance to deoxycholic acid and low pH

All 100 isolates of the collection were screened for resistance to deoxycholate and low pH, and antibiotic susceptibility was assessed. All tested isolates were able to grow in the presence of MRS broth containing 1.5% deoxycholic acid. Twenty-one percent of the strains were inhibited by 2% deoxycholic acid, and the rest were inhibited at 3%.

Additionally, all strains tolerate pH concentrations of 4; growth of 9% was inhibited at pH 3.5, and the rest

(91%) were inhibited at pH 3. Antibiotic resistance was not detected among in the *L. bulgaricus* collection, except for fusidic acid (87%), kanamycin (5%), moxifloxacin (2%), and tetracycline (1%) (table II).

A single isolate, *L. bulgaricus*-K98, belonging to the genogroup A, fully antibiotic susceptible, but able to tolerate 3% deoxycholate and pH 3, was finally selected. This strain was used to prepare homemade yogurt and given to volunteers with the aforementioned protocol.

Detection of *L. bulgaricus* K98 in faeces after yogurt intake

Before yogurt intake and at basal conditions, PCR for *L. bulgaricus* was consistently negative in DNA samples extracted from faecal samples of volunteers. After the daily ingestion of 500 g of the homemade yogurt during two consecutive weeks, positive amplicons with specific primers for *L. bulgaricus* was consistently obtained in the faecal samples of all 20 volunteers. Finally, a single colony showing an identical RAPD-PCR profile than the *L. bulgaricus* K98 yogurt strain was detected in one volunteer.

Changes in microbiota and SCFA after K98 yogurt intake

We first explored qualitative changes in microbiota using the universal primers in the DGGE experiments. A heterogeneous electrophoretic pattern of microbiota was observed among all volunteers, although this pattern was consistently stable when the two different faecal samples of each volunteer were compared (fig. 2). Yogurt intake did not produce any significant variation when the basal pattern was compared with that obtained at the end, even when specific LAB primers were applied for DGGE. On the other hand, basal samples of volunteers did not contain any band corresponding to *L. bulgaricus*, whereas a clear band emerged in all 20 samples obtained after yogurt intake (fig. 3).

In quantitative PCR, we found a significant increase of the total LAB and *C. perfringens* groups after yogurt intake ($p \leq 0.01$), whereas the total amount of the *Bacteroides-Prevotella-Porphyromonas* group decreased, being such decrease statistically significant for *B. vulgatus* ($p \leq 0.001$). Statistical significant changes were not observed for the *C. coccoides* group, although a decrease in the basal values was observed after the yogurt intake (fig. 4).

Then, we normalized the data obtained under basal conditions to classify the individuals in categories of low, normal and high density for each one of the bacterial groups (≤ 15 , 15-85, and $\geq 85\%$, respectively), and analyzed the qualitative changes after yogurt consumption. In the case of LAB and *C. perfringens*, the global

Table II
Antibiotic susceptibility results obtained in 100 *L. bulgaricus* isolates

Antimicrobial agent	Number of isolated with a MIC (mg/ml) of:											MIC ₅₀	MIC ₉₀	% resistance	
	≤0.06	0.1	0.25	0.5	1	2	4	8	16	32	64				≥128
Penicillin	87	3	3	4	1	2							≤0,06	0,25	0
Ampicillin	39	14	18	21	7	1							0,1	0,5	0
Gentamicin				4			14	19	33	25			16	32	0
Kanamycin				1		1	13	11	51	18		5	16	32	5
Streptomycin						35	5	25	35				8	16	0
Fusidic acid					8	4	1	7	1	11		8	64	64	87
Tetracycline				44	18	20	8	5		1			1	4	1
Vancomycine				19	38	39	4						2	2	0
Teicoplanin				85	13	2							0,5	1	0
Erythromycin	88	4	2	2	4								≤0,06	0,1	0
Cloranphenicol				29	7	8	9	47					4	8	0
Moxifloxacin				20	6	19	16	37			2		4	8	2
Linezolid				79	10	9	1	1					0,5	2	0
Quinupristin/dalfopristin	92	2	5	1									≤0,06	≤0,06	0

increase observed was at the expense of the low density group, whereas the *Bacteroides* decrease was most marked in the initial high density group.

Finally, considering the fecal concentration of all SCFAs, the total average value in the basal samples was 63.3 μmol/g, (increasing to 99.3 μmol/g after yogurt intake, and significant increases were observed for acetic (p < 0.01), butyric (p < 0.05) and 2-hydroxybutyric (p < 0.05) acids (fig. 5).

Discussion

In this work, we have explored the genetic diversity in a large collection of 100 *L. bulgaricus* strains originated in different homemade yogurts in rural Bulgarian areas, and screened with tolerance to environmental conditions of relevance in intestinal survival, such as low pH or presence of bile salts. Only the strain *L. bulgaricus* K98 was retained after the screening process, and was considered a good probiotic candidate.

The healthy properties of yogurt have traditionally been attributed to its biological effects promoted by the uptake of living bacteria responsible for milk fermentation, as these microorganisms are considered probiotics.¹³ This classical view implies the statement that viability of the yogurt bacteria is maintained in the gut after yogurt intake. Supporting this hypothesis, it can be expected that heat-treated yogurt, without viable organisms, might lack the beneficial effect of fresh preparations in lactose maldigestion³⁵. This view is also

corroborated by the *Codex Alimentarius* (.FAO. *Codex Standard for Fermented Milks*, 2002; 243-2003.), which recommends the name “yogurt” only for fermented milk with living bacteria. Our group has recently challenged the hypothesis of survival of the classic yogurt bacteria *L. bulgaricus* and *S. thermophilus* in the human gut, showing that yogurt effects may be considered as prebiotic rather than probiotic.^{15,18} On the other hand, in the present study, by using LAB DGGE primers which are more sensitive in detecting *L. bulgaricus*, we suggest that *L. bulgaricus* K-98 do not significantly reduces its cell density during its passage through the intestinal tract.

We have also shown significant changes in the microbiota quantitative analysis obtained after yogurt intake with *L. bulgaricus*-K98. After yogurt exposition, a clear reduction of the *Bacteroides* population with a concomitant increase of the LAB population was observed. These changes are considered as a healthy pattern as found in studies of microbiota with obese and diabetic patients,³⁶ as well as in other models of digestive tract diseases.³⁷⁻³⁹ It is of note that the study was designed to evaluate changes in the proportion of different bacterial groups after yogurt intake and results do not necessarily reflect the actual number of organisms in each of the groups. This number is certainly difficult to calculate, due to the heterogeneity in the number of rRNA copies inside each bacterial group.³² These quantitative modifications after *L. bulgaricus*-K98 yogurt consumption are similar as those detected after commercial yogurt intake,¹⁸ so future studies should assess whether higher quantities

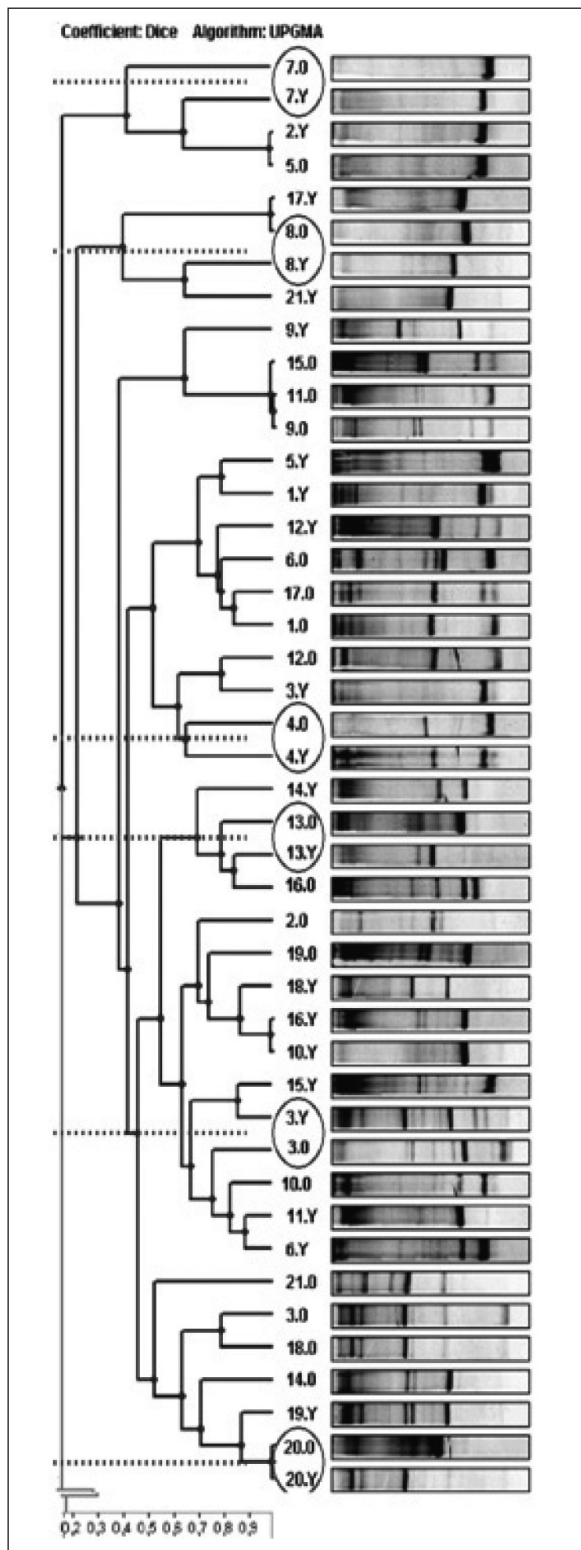


Fig. 2.—Dendrogram construction with the DGGE electrophoretic pattern using universal and LAB primers.

of this strain in yogurt or other preparations with this bacteria are more effective in inducing changes in microbiota than commercial yogurt. This is of clinical relevance, since both prebiotics and probiotics have

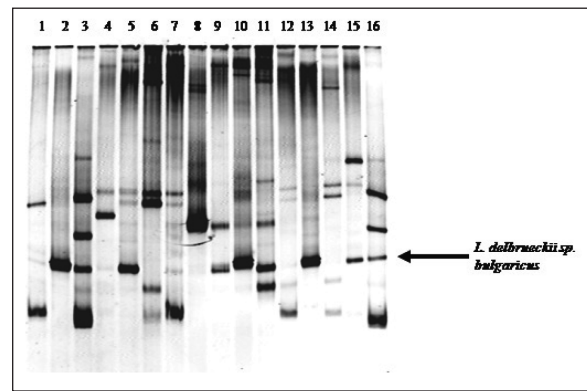


Fig. 3.—Representation of a DGGE gel using LAB primers. Lines 3 and 16: Molecular marker in which the band corresponds to *L. delbrueckii* subsp *bulgaricus*. is marked. The other are different volunteers in their two samples: line 1, 4, 6, 8, 10, 12, 14, and corresponded to the basal faecal sample, and lines 2, 5, 7, 9, 11, 13, and 15 to the faecal samples after yogurt exposure.

been found to be beneficial for several human conditions, but they differ in their modes of action and their overall effects.³⁹

In this sense, after a probiotic survives its passing through the intestine and manages to establish cell density-dependent effects within the microbiota, it exerts health-benefiting effects via many different mechanisms, such as inhibition of bacterial translocation, enhancement of mucosal barrier function, and signaling with the epithelium and immune system to modulate the inflammatory/immune response.⁴¹⁻⁴² Marked and parallel reduction in inflammation in the colon and cecum and in the production of pro-inflammatory cytokines by *Lactobacillus* has been demonstrated in experimental animal models,⁴³ and these anti-inflammatory effects could be mediated by bacterial DNA alone.⁴⁴ Therefore, the recovery of *Lactobacillus* DNA in all faecal samples of the studied individuals in our study underscores its relevance beyond the microbiota changes found.

The metabolic changes for the SCFA faecal concentration observed after yogurt consumption are probably related to quantitative changes in the bacterial composition. The majority of SCFA in the gut are derived from bacterial breakdown of complex carbohydrates, especially in the proximal bowel,⁴⁵ and the rate and amount of SCFA production depends on the species and amounts of microbiota present in the colon.⁴⁶ We found an increase for acetic, butyric and 2-hydroxy-butyric acids, which are the most abundant ones.^{5,35} This could be of clinical interest, as SCFA have shown anti-inflammatory effects and seem to have the potency to prevent infiltration of immune cells, to inhibit the proliferation and activation of T cells, and to prevent adhesion of antigen-presenting cells.⁴⁶ In agreement, a recent work has studied the effects of this probiotic in several immune parameters in healthy elderly volunteers.⁴⁷

In conclusion, we suggest that the intestinal survival of yogurt bacteria is strain-dependent, and we have

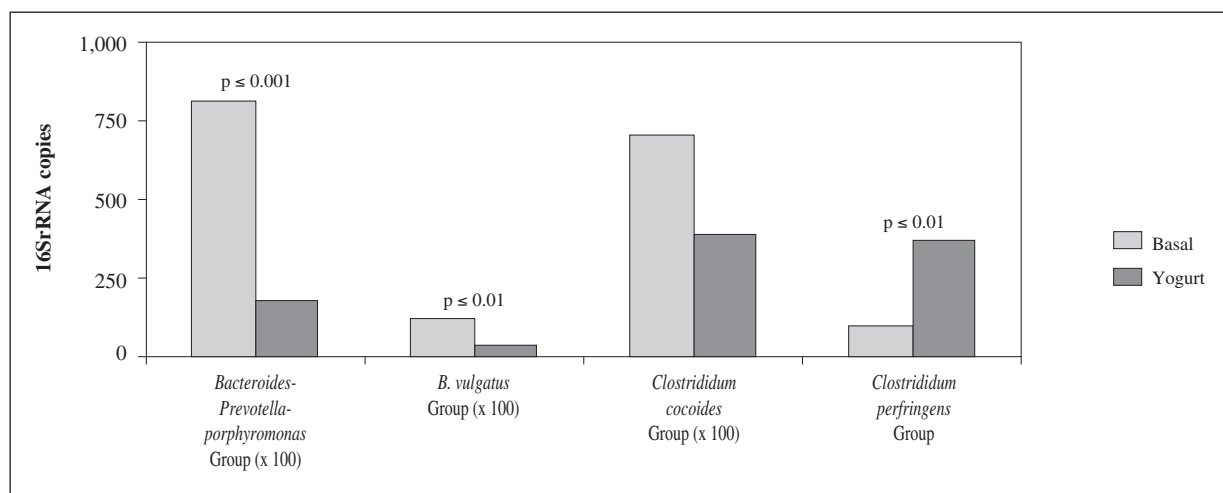


Fig. 4.—16S rRNA copies quantification by RT-PCR for the different groups of microorganisms in the basal samples and after yogurt consumption. Statistical differences are reflected for each group.

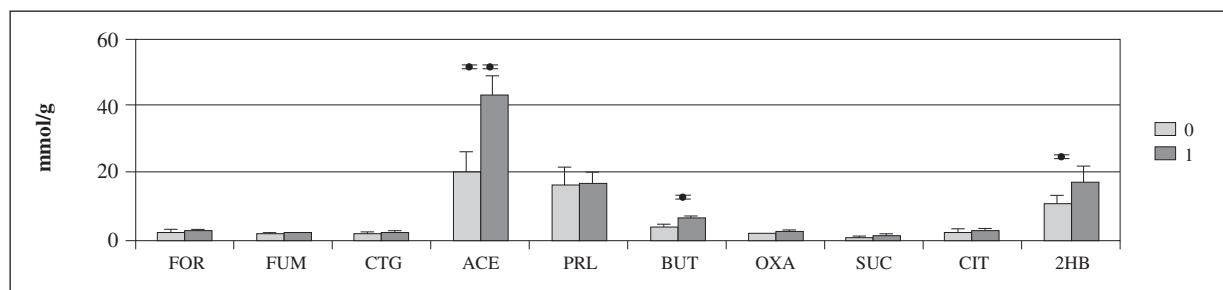


Fig. 5.—Variation of the different SCFA concentration in feces before (0) and after yogurt consumption (1). *Significant results ($p \leq 0.01$). FOR: formic acid; FUM: fumaric acid; CTG: ketoglutaric acid; ACE: acetic acid; PRL: propionic lactic acid; BUT: butyric acid; OXA: oxalic acid; SUC: succinic acid; CIT: citric acid; 2HB: 2-hydroxybutyric.

isolated a probiotic resistant strain to bile and high acidity, *L. delbrueckii subsp. bulgaricus*-K98. Qualitative and quantitative changes in the intestinal microbiota are found after ingestion of a homemade yogurt containing this strain, with a concomitant increase in faecal SCFA. Our findings support the interest in developing further studies providing different amounts of *L. delbrueckii subsp. bulgaricus*-K98, and should evaluate its clinical effects in human disease.

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