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ISOLATION AND IDENTIFICATION OF PROBIOTIC LACTIC ACID BACTERIA AND EXAMINATION OF THEIR TOLERANCE AGAINST STRESS FACTORS

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ABSTRACT. Probiotics are living microorganisms which have beneficial effect on the metabolism of the host organism when they pass into the large intestine. The number of living cells declines dramatically when they are going through the gastro-intestinal system, thus consumption of probiotic products with higher cell concentrations is recommended. Potential probiotic lactic acid bacterial strains were isolated from different yoghurts and food supplements and identified by traditional microbiological and molecular biological methods. According to the results the isolated strains belonged to Lactobacillus acidophilus, L. casei, L. rhamnosus, L. helveticus and L. delbrueckii subsp. bulgaricus. The tolerance of L. acidophilus, L. casei and L. rhamnosus against lactic acid, sodium chloride and sucrose was investigated by determination of the growing ability of the strains in a culture media containing different concentrations of these compounds. Among the tested strains L. rhamnosus had similar growth rate as L. casei in lactic acid and media sucrose containing, while it showed a better tolerance for sodium chloride. L. acidophilus strain proved to be more sensitive for lactic acid and sucrose as the above mentioned strains.

Keywords: *probiotics, isolation, identification, stress tolerance*

INTRODUCTION

The concept of probiotics is not new and probiotic products have been consumed by humans in the form of fermented foods for thousands of years. Health benefits of probiotics have also been long known because Hippocrates and other scientists in the early ages reported that fermented milk could cure some disorders of the digestive system. Even Biblical scriptures mentioned the use of probiotics when treating body ailments. It was in 1907 when Elie Metchnikoff first proposed the concept of probiotics as it is known today. He observed that consuming large amounts of fermented milk products

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containing lactobacilli prolonged the life of humans and he gave the first scientific explanation for the beneficial effects of lactic acid bacteria being present in fermented milk. Today it is accepted that the daily intake of these probiotics contributes to improving and maintaining a well balanced intestinal biota and prevents gastrointestinal disorders (Ranadheera et al., 2010). According to the definition of the World Health Organization probiotics are living microorganisms which - when administered in adequate amounts - provide a health benefit to the host. Beneficial effects are manifested by, for example, the inhibition of pathogenic bacteria, the synthesis of vitamin B and bacteriocins in the colon, by lowering of ammonia level of the blood, the absorption of cholesterol, and the inhibition of tumour formation (Capela et al., 2005; Kos et al., 2007).

An important question to be answered is how much the "adequate amounts" of probiotics are. The level of viable microorganisms in probiotic foods has to be at least 10^7 cfu/g to provide better efficacy in regulating beneficial effects (Ranadheera et al., 2010). In most cases the number of viable microorganisms in probiotic products decreases dramatically during the storage process because of the physico-chemical properties (such as low pH, buffering capacity, etc.) of food carriers used for probiotic delivery. These physical and chemical characteristics present significant stress factors that influence the survival of probiotic microorganisms (Gupta and Abu-Ghannam, 2012, Ranadheera et al., 2010).

The increasing application of probiotic cultures in food products underlines the need for an exact identification of these beneficial microorganisms and distinguishing them from the original microbial population of the digestive system. Moreover, certain probiotic activities are not only species- but also strain-specific; therefore, the identification of probiotic bacteria is indispensable at least at species level. Current techniques used for the identification and typing of microorganisms comprise multiple DNA-based methods such as pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) analysis, ribotyping and species-specific PCR (Kos et al., 2007).

The majority of known probiotic bacteria belong to *Lactobacillus* (e.g. *L. bulgaricus, L. acidophilus, L. casei, L. helveticus, L. lactis, L. salivarius, L. plantarum*) or *Bifidobacterium* spp., but certain strains of *Bacillus, Streptococcus*, *Pediococcus* and *Enterococcus* spp. also proved to have probiotic effects (Musikasang et al., 2009).

The main purpose of our research was to isolate and identify potential probiotic lactic acid bacteria and determine their susceptibility against the stress factors typical for the different food environments. The isolation, identification and examination of the growth of probiotic lactic acid bacteria are significant milestones of the main objectives, involved in the title of this paper as well.

RESULTS AND DISCUSSIONS

Isolation of probiotic bacteria

Samples were taken from three commercially fermented dairy products (yoghurts) and two food supplements (a synbiotic product for teenagers and adults, and a probiotic infant formula). From the samples which were inoculated and cultivated on deMan-Rogosa Sharpe (MRS) agar plates supplemented with the antifungal antibiotic Nystatin, altogether twenty-six colonies were isolated that had differences in colony morphology (Table 1.). From the probiotic yoghurts type 1 and type 2, four and two bacteria were isolated, respectively. Two strains were isolated from normal yoghurts, while the majority of the isolates originated from synbiotic capsules (12 strains) and probiotic infant formula (6 strains). Based on microscopic cell morphology examination four strains (marked with asterix in Tabel 1.) showed typical *Bifidobacterium* properties, thus they were not used for further investigations.

Table 1. The sources and codes of isolated strains

Nr.	Products used for sampling	Code of isolates
1	Probiotic yoghurt, type 1	VA1
2	Probiotic yoghurt, type 1	VA2
3	Probiotic yoghurt, type 1	VA3
4	Probiotic yoghurt, type 1	VA4
5	Normal yoghurt	EMJ1
6	Normal yoghurt	EMJ2
7	Probiotic yoghurt, type 2	BJP1
8	Probiotic yoghurt, type 2	BJP2
9	Synbiotic capsule	PB1
10	Synbiotic capsule	PB2
11	Synbiotic capsule	PB3
12	Synbiotic capsule	PB4
13	Synbiotic capsule	PB5
14	Synbiotic capsule	PB6
15	Synbiotic capsule	PB7
16	Synbiotic capsule	PB8
17	Synbiotic capsule	PB9
18	Synbiotic capsule	PB10
19	Synbiotic capsule	PB11
20	Synbiotic capsule	PB12
21	Probiotic infant formula	BPK1*
22	Probiotic infant formula	BPK2*
23	Probiotic infant formula	BPK3*
24	Probiotic infant formula	BPK4*
25	Probiotic infant formula	BPK5
26	Probiotic infant formula	BPK6

*- strains proved as bifidobacteria

Gram staining and catalase test

All the examined isolates (22) stained positively with Gram staining procedure and gave negative catalase reaction, which are the typical characteristics of lactic acid bacteria (Salminen et al., 2004).

Differentiation of the isolates on mMRS-BPB chromogenic medium

Since anaerobic conditions were used during the isolation of bacteria the *Bifidobacterium* species - if present in the products - could also form colonies on MRS agar. As species of *Bifidobacterum* are also Gram-positive and catalase-negative bacteria, they could not be distinguished from *Lactobacillus* species using Gram staining and catalase test. However, by the application of the modified deMan-Rogosa Sharpe agar supplemented with bromophenol blue (mMRS-BPB) as culturing medium under anaerobic conditions, the differentiation of these two genera was feasible (Lee and Lee, 2008). Moreover, this medium enabled the differentiation of the most important *Lactobacillus* species based on the marked differences in colony morphology. Figure 1. shows the differences between colony morphology of *Lactobacillus rhamnosus* PB10 and *Bifidobacterium animalis* subsp. *lactis* BPK6 strains.

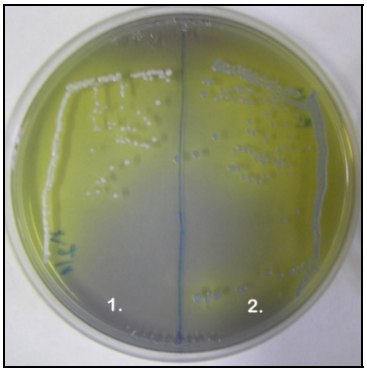

Figure 1. Colonies of *Lactobacillus rhamnosus* PB10 (1.) and *Bifidobacterium animalis* subsp. *lactis* BPK6 (2.) on mMRS-BPB agar grown under anaerobic conditions for 48 hours

Identification and typing of bacterial isolates by molecular biological methods

After differentiating the isolates on mMRS-BPB agar the selected isolates were cultivated in MRS broth and the cellular DNA was extracted by the method described by Hoffman and Winston (1987).

The isolates were genotyped by using the Randomly Amplified Polymorphic DNA (RAPD-PCR) method with the application of the M13 primer (Rossetti and Giraffa, 2005). The PCR products were separated by agarose gel electrophoresis and visualized by ethidium-bromide staining. Figure 2. shows an example for RAPD patterns (fingerprints) of the bacterium isolates. Based on the comparison of these fingerprints with each other and with that of the reference strains (BC2 - *Lactobacillus acidophilus* N2, BC3 - *Lactobacillus delbrueckii* subsp. *bulgaricus* B397, BC4 – *Lactobacillus rhamnosus* VT1, BE – *Lactobacillus sakei* DSM 20017) the clonal identity of the isolates was determined.

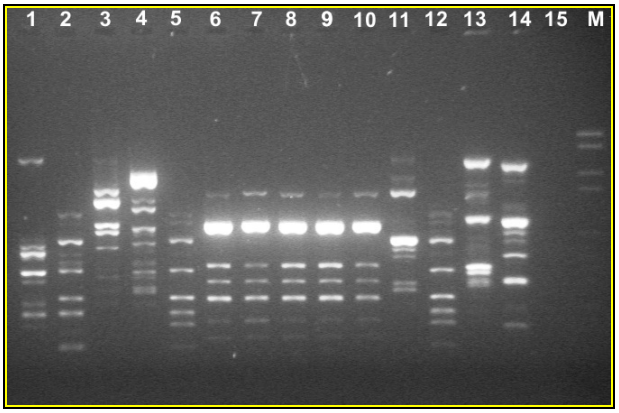

Figure 2. Example of RAPD-PCR patterns on agarose gel. 1- BC2; 2- BC3; 3- BC4; 4- BE; 5- PB1; 6- PB2; 7- PB3; 8- PB4; 9- PB5; 10- PB6; 11- PB7; 12- PB8; 13- BPK5; 14- BPK5; 15- control; M-molecular weight marker

It was found that the bacterium isolates belonged to eight different clusters (Table 2). Moreover, an evaluation of the results made it possible to separate the isolates originated from the same products into different groups (for instance: VA2, VA4 - from probiotic yoghurt type 1; PB3, PB8, PB10 - from symbiotic capsule; BPK6, BPK5 - from infant formula; and EMJ1 - from a normal yoghurt).

Single representative isolates were selected from each cluster, with the exception of cluster 1 where two isolates were elected from. In the latter case, slight differences in the RAPD patterns of the VA2 and BJP2 isolates were recognised.

Identification of the isolates at species level was done by sequence analysis. 16S rDNA sequences flanked by the 27f-519r primer pair (Thanantong et al., 2006) were amplified by a PCR reaction. The amplification resulted in PCR products of approximately the same size; however, in case of BPK5 isolate the amplicon proved to be smaller in size (Figure 3.).

Table 2. Eight different similarity clusters of the RAPD-PCR analysis

	Clusters							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Code of the Isolates	VA1 VA2 VA3 BJP1 BJP2	VA4	PB2 PB3 PB4 PB5 PB6 PB 9	PB1 PB 8 PB 11 PB12	PB7 PB10	BPK 6	PBK 5	EMJ1 EMJ2

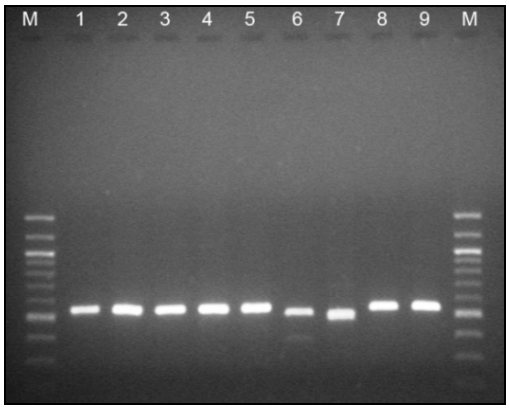

Figure 3. Agarose gel electrophoresis of the 16S rDNA-specific PCR products. M- molecular weight marker; 1- VA2; 2- VA4; 3- PB3; 4- PB8; 5- PB10; 6- BPK6; 7- BPK5; 8- BJP2; 9- EMJ1

Table 3. List of species identified by DNA sequencing

Strains	**Results of identification**	**Similarity index**	**RAPD cluster**
BJP2	*Lactobacillus acidophilus*	100%	1
VA2	*Lactobacillus acidophilus*	100%	1
VA4	*Lactobacillus casei*	99%	2
PB3	*Lactobacillus helveticus*	99%	3
PB8	*Lactobacillus casei*	99%	4
PB10	*Lactobacillus rhamnosus*	99%	5
BPK6	*Bifidobacterium animalis* subsp. *lactis*	99%	6
BPK5	Could not be evaluated	-	7
EMJ1	*Lactobacillus delbrueckii* subsp. *bulgaricus*	99%	8

The PCR products obtained were sequenced and the sequences were aligned to the 16S rDNA bacterial sequences found in the GeneBank. The species identity of our isolates is indicated in Table 3.

Based on the results of RAPD analysis done by the application of M13 primer and 16S rDNA sequencing it can be concluded that the same strains of *Lactobacillus acidophilus* could be isolated from probiotic yoghurt type 1 and type 2 that are products of different companies.

Examination of growing ability of the strains under different stress conditions

Determination of cell concentrations was performed by measuring the optical density (OD) at 600 nm in different time points. Growth curves are illustrated on Figures 4-12.

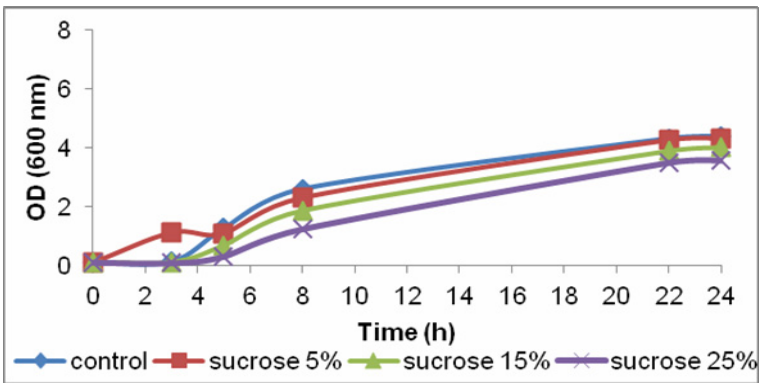

Figure 4. The growth curves of *L. acidophilus* VA2 in different sucrose concentrations (pH 5.7)

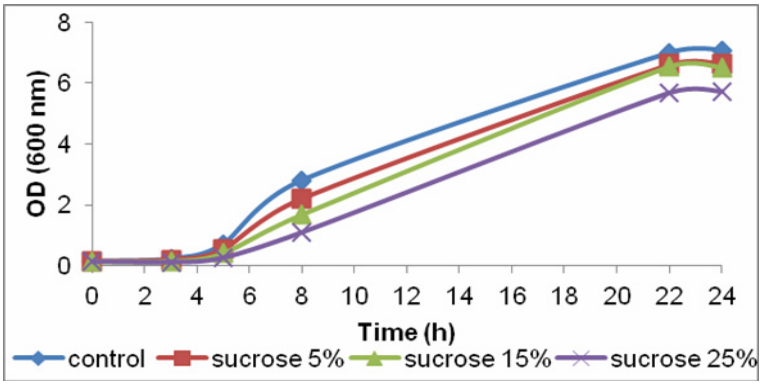

Figure 5. The growth curves of *L. rhamnosus* PB10 in different sucrose concentrations (pH 5.7)

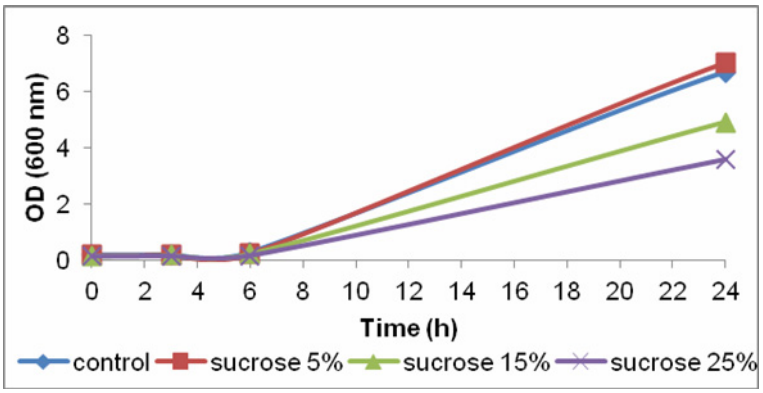

Figure 6. The growth curves of *L. casei* PB8 in different sucrose concentrations (pH 5.7)

Comparing the results of growing ability under different sucrose concentrations – as shown in Figures 4-6 – it can be assessed as a general rule that the higher amount of sucrose in the culture media presents an important stress factor. Moreover, there is an observable difference between the three strains belonging to different species, namely that *L. casei* and *L. rhamnosus* had approximately the same growth kinetic, but *L. acidophilus* showed a declining growth rate.

The sodium chloride tolerance (Figures 7-9) of these three strains showed that the greatest growth ability could be detected in the case of *L. rhamnosus*, followed by the *L. acidophilus* strain. Interestingly, *L. casei* showed the highest sensitivity against this stress factor.

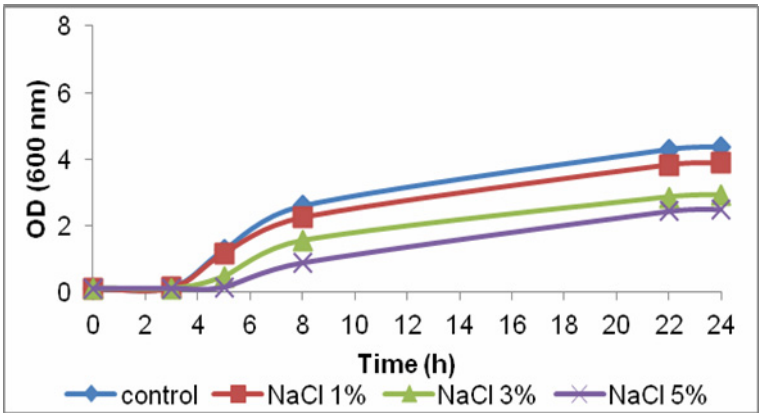

Figure 7. The growth curves of *L. acidophilus* VA2 in different NaCl concentrations (pH 5.7)

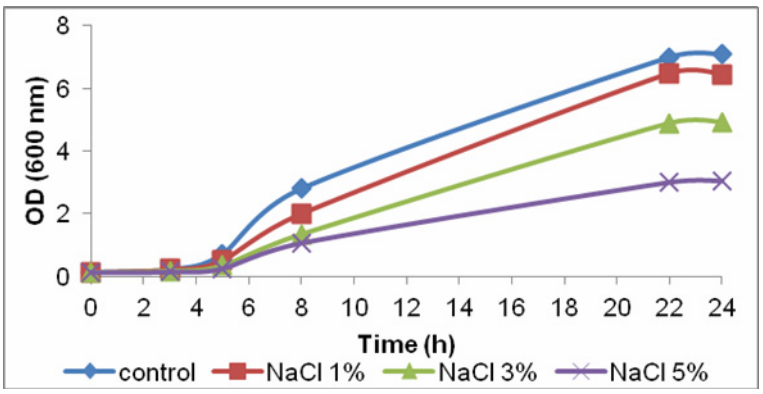

Figure 8. The growth curves of *L. rhamnosus* PB10 in different NaCl concentrations (pH 5.7)

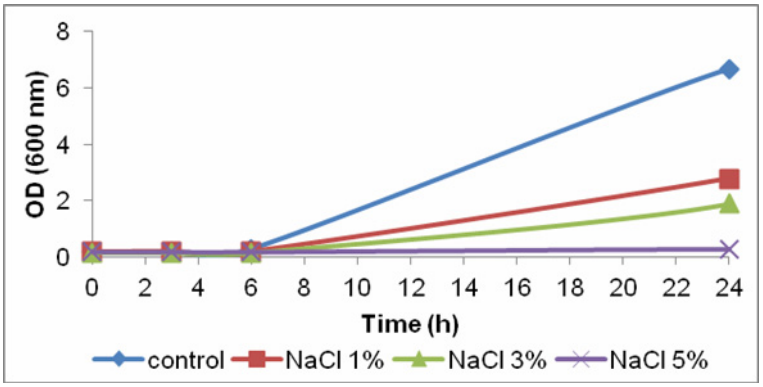

Figure 9. The growth curves of *L. casei* PB8 in different NaCl concentrations (pH 5.7)

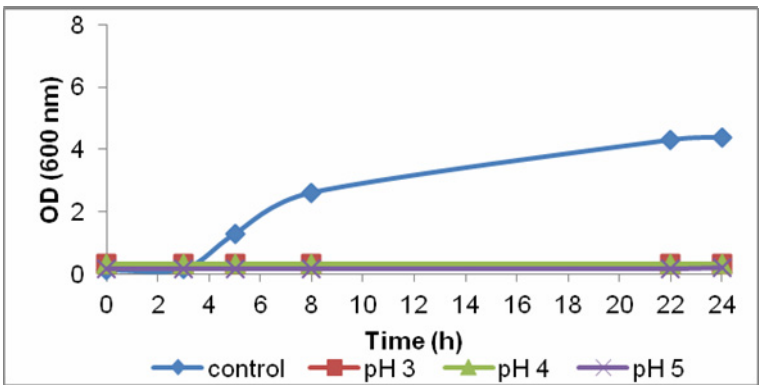

Figure 10. The growth curves of *L. acidophilus* VA2 at different pH values. pH of the control was 5.7

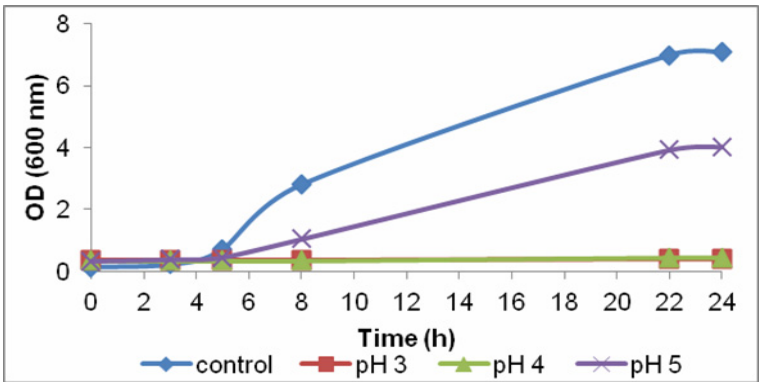

Figure 11. The growth curves of *L. rhamnosus* PB10 at different pH values. pH of the control was 5.7

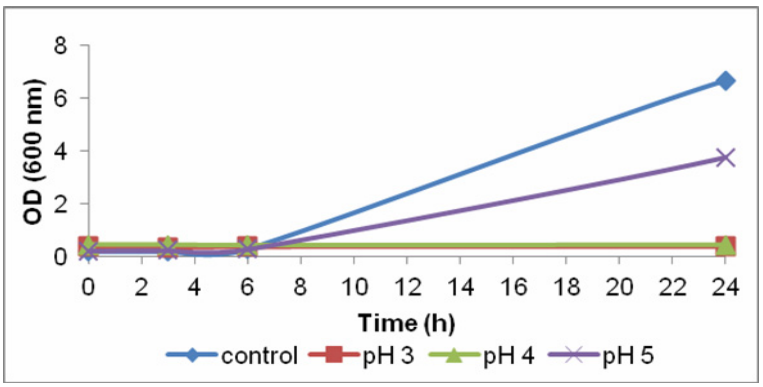

Figure 12. The growth curves of *L. casei* PB8 at different pH values. pH of the control was 5.7

According to the results illustrated on Figures 9-12 lactic acid which was used for the regulation of pH had the most significant growth inhibitory effect among the three investigated stress factors. The growth of the strains could be observed only above pH 4, and there was a similar growth tendency observed in the case of *L. casei* and *L. rhamnosus*. However, the weak acid tolerance of *L. acidophilus* was surprising, because this species is known as having a good acid tolerance.

CONCLUSIONS

Potential probiotic lactic acid bacteria were isolated and discriminated from the bifidiobacteria and each other by using the chromogenic MRS medium mMRS-BPB. Based on the RAPD-PCR typing followed by molecular

identification of the isolates five *Lactobacillus* and one *Bifidiobacterium* species were identified. Three of them (*Lactobacillus acidophilus* VA2, *L. casei* PB8 and *L. rhamnosus* PB10) were selected and their growing abilities were investigated in culture media containing different concentrations of lactic acid, sodium chloride and sucrose. Comparing the sensitivity of the three strains against these stressing factors we can conclude that the *L. rhamnosus* had a similar growth rate as *L. casei* in media containing lactic acid or sucrose, while it showed better tolerance for sodium chloride than the other two species. *L. acidophilus* proved to be sensitive to sucrose and lactic acid but it had a better tolerance against sodium chloride, than *L. casei*. As the final conclusion it can be said that *L. rhamnosus* had the best tolerance against the investigated stress conditions, which suggests the future use of this species for microencapsulation or as a probiotic supplement in various food products.

EXPERIMENTAL SECTION

Isolation of probiotic bacteria

An initial suspension of the collected samples (yoghurts and food supplements) was made in salt-peptone solution (0.85% NaCl and 0.1% Bacto-peptone). After this step ten-fold dilution series were prepared from which 0.1 ml quantities were spread on MRS agar (Merck 1.10661.0500) containing 0.01% Nystatin. After incubation for 48 hours at 37°C under anaerobic conditions (in anaerobic jar containing anaerobic sachet - Anaerocult A, Merck 1.13829.0001) pure cultures of the isolated colonies having different morphological properties were made using MRS agar plates.

Gram staining and catalase test

From the pure cultures of the isolates cultivated in MRS broth, Gram staining and catalase test were performed as it is described in the Bacteriological Analytical Manual (BAM, 1998). The catalase test was done by dropping 3% H_2O_2 solution to the bacterial colonies grown on agar plates, and gas formation was checked in each case.

Differentiation on mMRS-BPB agar

The modified deMan-Rogosa Sharpe agar containing bromophenol blue (Lee and Lee, 2008) was prepared in the following way: required quantity of deMan-Rogosa Sharpe agar granule and L-cysteine-HCl (final conc. 0.05%) were dissolved in 1000 ml of distilled water and bromophenol blue (BPB) was added to the final concentration of 0.002%. The pH was adjusted to 6.5 ± 0.2 and then it was autoclaved. The isolates were grown on this agar under anaerobic conditions for two days at 37°C.

RAPD-PCR analysis

The selected isolates were cultivated in MRS broth for 24 hours at 37°C under aerobic conditions. The DNA was extracted from 1 ml cultures as it is described by Hoffman and Winston (1987).

Genomic DNA from each isolate was used as a template for PCR fingerprinting (RAPD-PCR) using the M13 minisatellite primer (Rossetti and Giraffa, 2005, Albesharat et al., 2011). Amplification reactions were performed according to the optimised protocol: denaturation at 95°C for 5 min was followed by 35 cycles of 95°C for 30 sec (denaturing), 40°C for 30 sec (annealing) and 72°C for 1.5 min (extension). Final extension was carried out at 72°C for 5 min. Reactions were carried out in 25 µl final volume. The reaction mixture contained 10x DNA polymerase puffer, 2.5 µmol of M13 primer, 2 µl of extracted DNA, 1.25 mmol $MgCl_2$, 0.3 U of *Taq* polymerase (DyNAzyme™ II, Finnzymes) and 0.3 mmol of dNTP. PCR amplified DNAs were separated by gel electrophoresis (1.2 V cm^{-1}) using 1.5% (w/v) agarose gel and visualised by staining with ethidium bromide. DNA molecular size marker MW VI. (Boehringer Mannheim, Germany) was used to estimate the size of the amplicons.

Molecular identification of bacterium isolates

Specific PCR targeting the 16S rDNA (Pang et al., 2011) was used for the amplification of an approximately 500 bp long sequence by the 27f and 519r primers (Thanantong et al., 2006). The reaction was carried out according to the following protocol: initial denaturation for 5 minutes at 95°C was followed by 35 cycles of 95°C for 30 sec (denaturing), 55°C for 1 min (annealing) and 72°C for 1 min (extension). Final extension was carried out at 72°C for 3 min. Reactions were carried out in a final volume of 25 µl. The reaction mixture contained 10x DNA polymerase puffer, 2.5 µl of 27f and 519r primers (2 pmol/µl for each), 1 µl of template DNA, 1 mmol $MgCl_2$, 0.3 U of *Taq* polymerase (DyNAzyme™II, Finnzymes) and 0.375 mmol of dNTP. PCR amplicons were visualised by ethidium bromide staining after agarose gel electrophoresis (1.2 V cm^{-1}) in 1% (w/v) agarose gel.

Sequencing of the PCR amplified region (500 bp) of the 16S rDNA was applied to identify the isolates at species level. DNA sequencing was carried out by the BayGen Institute, Szeged, Hungary. Identification of the isolates was performed by comparing the obtained nucleotide sequences with that of known bacteria deposited in the GeneBank using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Determination of the growth curves

Three strains belonging to *Lactobacillus acidophilus* VA2, *L. casei* PB8 and *L. rhamnosus* PB10 were selected for further investigations. These strains were cultivated under aerobic conditions at 37°C for 24 hours in MRS

broth. From these cultures 1 ml was inoculated into MRS broth as a control having initial pH 5.7 and in MRS broth with pH 3.0, 4.0, 5.0 adjusted by lactic acid as well as different concentrations of NaCl (1%, 3% and 5%) or sucrose (5%, 15% and 25%). During the incubation of the strains the optical density (OD) was measured at 600 nm (Rao et al, 2004, Ayad et al., 2006).

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