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PHYSIOLOGICAL AND METABOLIC RESPONSES OF FUNCTIONAL LACTIC ACID BACTERIA TO STRESS FACTORS

Iulia-Roxana ȘTEFAN[1,2], Călina-Petruța CORNEA[1], Silvia-Simona GROSU-TUDOR[2], Medana ZAMFIR[2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania; Email: pccornea@yahoo.com
[2]Institute of Biology Bucharest, Romanian Academy, 296 Splaiul Independentei, Bucharest, Romania; Email: iulia.stefan@ibiol.ro

Corresponding author email: pccornea@yahoo.com

Abstract

In our study, six functional lactic acid bacterium strains (able to produce bacteriocins, exopolysaccharides, or S-layer proteins) were subjected to different stress conditions in order to evaluate their capacity to adapt to harsh environments. They were cultivated at different temperatures, below and above their optimal growth temperatures. Moreover, they were grown in presence of bile salts or NaCl, or in acidic media. Total proteins, lactic acid production, enzymatic activities and synthesis of the metabolites of biotechnological interest were evaluated for all growth conditions. Over expression of some proteins was detected in the electrophoretic pattern of the total protein extracts from the stressed cultures compared to the control cultures. Enzymatic activities and lactic acid production were, in general, directly related with growth. The tested strains maintained their capacity to synthesize the assayed metabolites under almost all stress conditions. Bacteriocin production of two strains and exopolysaccharides production of both Leuconostoc strains were correlated with growth, while the S-layer production and the bacteriocin production by strain L. helveticus 34.9 were enhanced under stress conditions.

Key words*: bacteriocins, enzymes, exopolysaccharides, lactic acid bacteria, S-layer, stress.*

Abbreviations:
NAD - Nicotinamide adenine dinucleotide
NADP - Nicotinamide adenine dinucleotide phosphate
NBT - Nitrobluetetrazolium
PMS - Phenazinemethosulfate
MTT - 3-(4, 5-dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium bromide

INTRODUCTION

Lactic acid bacteria (LAB) constitute a group of important microorganisms in food industry. They have a major contribution to the organoleptic qualities and final texture of the fermented food products. Naturally fermented products contain both functional and non-functional microorganisms (Tamang et al., 2016). Functional bacteria have an important role in food and feed industry. Firstly, they can enhance the bio-availability of nutrients by transforming the chemical components of raw vegetal/animal ingredients during food fermentation. Secondly, functional bacteria provide characteristics that make the final product valuable, such as polysaccharides important for texture, different proteins (i.e. surface layer proteins - S-layer), enzymes, vitamins and other compounds important for the consumer's health (i.e. antimicrobial substances) (Tamang, 2015; Farhad et al., 2010; Bourdichon et al., 2012; Thapa and Tamang, 2015). The microorganisms used as starter culture in fermentations are selected due to their impact on texture and aroma formation, as well as for functional properties and the quality of the final products (van de Guchte et al., 2002; Millsand De Vuyst, 2004). During the biotechnological processes of obtaining fermented food products, the bacteria have to adapt to different media conditions, such as presence of NaCl and temperatures below and above their optimal growth conditions. Furthermore, after ingestion, probiotic bacteria encounter harsh conditions in the gastro-intestinal tract, such as the presence of bile salts or low pH, to which they must resist in

order to survive and colonize the intestinal mucosa (Dunne et al., 1999). Bacteria respond differently to different types of stress conditions, depending on the species, strain and type of stress (Serrazanetti et al., 2009). One of the mechanisms developed by bacteria to adapt to environmental challenges is the synthesis of different proteins involved in ribosome stability, temperature sensing and ribosomal functions (De Angelis and Gobetti, 2004) such as chaperones and Heat Shock Proteins (HSP). Also, LAB are capable of inducing an ATR (acid tolerance response) as response to mild acid treatment. This system includes pH homeostasis, protection and repair mechanisms. Other mechanisms of resistance to acidic stress include ATPase triggered proton pumping, malo-lactic fermentation, and decarboxylation reactions among others (Serrazanetti et al., 2009). Moreover, in food industry, LAB can be exposed to osmotic stress when important quantities of NaCl are added to the product. Therefore, significant metabolic changes can occur in this case, including the induction of groEL, groESand dnaK stress proteins and membrane associated proteins (FtsH and HtrA) (van de Guchte et al., 2002).

On the other hand, the stress response to the presence of bile salts was less studied and, therefore, the mechanisms of adaptation and survival in this condition are not fully described. Recent studies showed several genes and molecules involved in this process, such as genes encoding muramidases among others (Lebeer et al., 2008; Bron et al., 2004).

Adaptation to stress enables a better survival of bacteria and also different performances in a system. Although stress responses originally evolved to benefit the bacterium, many of the metabolic changes occurring in stressful environments can be exploited in fermented foods. Understanding the mechanism involved in stress adaptation is important for selecting the best strains for a particular product.

In our study, six bacterial strains isolated from raw or fermented foods were grown under some stress condition that can occur during industrial processing or in the gastro-intestinal tract after ingestion. Our main objective was to assess the metabolic responses and changes in the functional properties of these strains when cultivated with low temperature, body temperature or higher, low pH, and in the presence of bile salts (BS) and sodium chloride, respectively.

MATERIALS AND METHODS

LAB strains and growth conditions

In our study, six functional lactic acid bacteria (LAB) strains isolated in our laboratory from different sources (Table 1) were tested in different growth conditions (Table 2) in order to evaluate their responses to stress factors.

Table 1. Selected bacterial strains and their functional properties

Bacterial strain	Functional property	Source of isolation
Lactococcus lactis 19.3	Bacteriocins	cows milk
Lactobacillus plantarum 26.1	Bacteriocins	sour cream
Leuconostoc mesenteroides P109	exopolysaccharides	bell pepper
Leuconostoc mesenteroides P124	exopolysaccharides	yellow beans
Lactobacillus brevis FV403	S-layer	brine (mixed vegetables)
Lactobacillus helveticus 34.9	S-layer, bacteriocins	fermented milk

Table 2. Growth conditions used in the present study

	19.3	**26.1**	**P109**	**P124**	**34.9**	**403**
Low temp.	20°C	20°C	15°C	15°C	28°C	15°C
Optim. temp.	28°C	28°C	28°C	28°C	37°C	37°C
High temp.	37°C	37°C	37°C	37°C	42°C	42°C
% NaCl	2%	5%	5%	5%	2%	5%
% BS	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%
Initial pH	5.5	4.5	4.5	4.5	4.5	4.5

The strains preserved at -80°C in MRS medium (de Man et al., 1960) with 25% glycerol have been subcultured twice in fresh MRS broth prior to use. *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901^T was used as indicator for the antibacterial properties of the targeted strains. Solid MRS was obtained by supplementing the broth with 1.5% agar, while top-layer MRS was obtained with 0.7% agar. For exopolysaccharides (EPS) quantification, the two producing strains were grown in filtered MRS (MRS_f) medium prepared according to Van der Meulen et al. (2007). All the incubations were carried on for 24 h.

Growth evaluation

Growth/survival was evaluated by measuring the optical density (OD) of the culture at 600

nm and the final pH. Moreover, the concentration of lactic acid (L.A.) produced by the strains in all the growth conditions was determined by HPLC using a Hamilton PRP-X 300 column (7 µm, 4.1 x 150 mm) based on ion exclusion (temperature of 60°C, elution with 2.5 mM H_2SO_4 at 0.5 ml/min).

Protein extraction and quantitative determination of enzymatic activities

Fifty milliliters of fresh 24 h cultures were centrifuged (13 000 *x g*, 10 min., 4°C), the cells were washed twice with Tris-HCl 0.1 M, pH 7.5, and resuspended in the same buffer. Afterwards, the proteins were extracted with FastPrep-24™ 5G Homogenizer (MP Biomedicals, USA) with the preset protocol for *Lactococcus lactis*. After a final centrifugation to eliminate the cell debris, protein content was measured with Bradford method (results not shown) (Bradford, 1976). The activities of lactate-dehydrogenase (LDH), alcohol-dehydrogenase (ADH), malate-dehydrogenase (MDH) and super-oxide dismutase (SOD) were quantitatively assessed according to spectrophotometric methods described by de Vallee and Hoch (1993) or Winterbourn et al. (1975).

Gel-electrophoresis of protein extract

One-dimensional Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis (SDS-PAGE) of the whole protein extracts was conducted at a constant voltage of 90 V in the stacking gel and 180 V in the running gelin a Biometra Minigel Twin (Biometra, Germany) apparatus. Sample preparation and gels were done according to Laemmli (1970). Concentrations of 4%, and 12% polyacrylamide were used for stacking and running gel, respectively. Broad range protein marker (Promega, USA) was used as reference and the gels were stained with Coomassie Briliant Blue R 250 (Carl Roth GmbH, Germany).

Electrophoresis of intracellular enzymes

The changes and variations of some intracellular enzymes (LDH, ADH, MDH, and SOD) and isoenzymes patterns of the bacterial strains cultivated under stress conditions were evaluated by one dimensional electrophoresis of the whole protein extracts as described previously, except that gels and buffers used were SDS-free. After migration, the gels were washed gently with ultrapure water and stained with specific mixtures for every enzyme.

For LDH detection, the blue bands appeared after 2 hours of incubation in the dark in a mixture containing Tris A buffer (0.2 M Tris, 1 mM EDTA, pH 8.0), 0.5 ML - or D, L - lactic acid (Merck), and 1% solutions in water of NAD^+, NBT, and PMS (all three from Sigma - Aldrich), respectively (Whitt, 1970).

For ADH detection, the gels were immersed in a solution of Tris A buffer, 0.5 M $MgCl_2$, ethanol (95°), 1% solutions in water of NAD^+, NBT, MTT (Sigma-Aldrich), and PMS, respectively and incubated in the dark until bands appeared (Tanksley, 1979).

The detection of MDH was made by incubating the gels in the dark in a solution of Tris A buffer, 0.5 M $MgCl_2$, and 1% solutions in water of NAD^+, NBT, and PMS, until blue bands appeared.

For detection of SOD isoenzymes, the gels were immersed in a solution containing Tris A buffer, 0.5 $MgCl_2$, NAD^+, NBT, and PMS (all as 1% solution in water). The incubation was carried at room temperature, under a neon tube. SODs appear as light bands on a dark background.

EPS isolation and quantification

The isolation and quantification of the synthetized EPS were performed as described by Degeest and De Vuyst (1999). Briefly, 50 ml of MRS_f adjusted to different conditions were inoculated with 2% (v/v) overnight (ON) culture of the EPS - producing strains. After incubation, the cultures were treated with 10% (final concentration) trichloroacetic acid (TCA) in order to remove the proteins.

Afterwards, the protein-freesupernatants have been mixed with cool acetone and let ON to precipitate the EPS. The precipitated EPS were dried for 48 h at 37°C and weighed in order to determine the polymer dry mass (PDM).

Antibacterial properties

The antibacterial activities of *Lactococcus lactis* 19.3, *Lactobacillus plantarum* 26.1 and *Lactobacillus brevis* 34.9 bacteriocins were tested and quantified by a modified critical dilution method described by De Vuyst et al. (1996). Briefly, the ON cultures were centrifuged (13 000 *x g*, 10 min, 4°C) and two-fold dilutions of each supernatant were spotted (10 µl) on fresh lawns of top-layer MRS

inoculated with *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901^T as indicator strain.

S-layer proteins detection

In order to evaluate the influence of the growth conditions on the S-layer proteins synthesis, SDS-PAGE was performed as described above. ON cultures obtained under different stress conditions were centrifuged. Cells were resuspended in sample buffer (Laemmli, 1970) and boiled for five minutes to extract the proteins. The supernatant was used for SDS-PAGE.

Statistical analysis

All the experiments were carried out in triplicate. The quantitative determinations are shown as mean ± standard deviation (SD) and the calculations were performed using Microsoft Excel software (Microsoft Corp.).

RESULTS AND DISCUSSIONS

During industrial processes or during the passage through the gastro-intestinal tract, (probiotic) LAB are exposed to various stress conditions and therefore, in order to resist to all the challenges, functional bacteria have developed different mechanisms of adaptation to these stressful conditions. Understanding these mechanisms is very important from a scientific and technological point of view.

Our study shows different responses of the selected functional LAB to stress factors (reduced/increased growth temperature, low initial pH, bile salts or NaCl) in terms of growth and production of several metabolites important for cell protection (S-layer), food texture (EPS), and health (bacteriocins, enzymes).

Bacterial growth under stress conditions

The growth parameters measured after 24 h of incubation are presented in Table 3. All strains grew very well under their optimal conditions. Moreover, the higher and lower temperatures did not affect significantly the growth, OD and pH values being similar with the ones for optimal temperature. Strains 19.3, 26.1 and FV403 showed a very good growth in the presence of NaCl and in acidic growth medium, with OD values similar with the ones for optimal conditions.

Table 3. Growth parameters under different growth conditions of *Lact. lactis* 19.3, *Lb. fermentum* 26.1, *Leuc. mesenteroides* P109 and P124, *Lb. helveticus* 34.9, and *Lb. brevis* FV403

		OD_{600nm}	Final pH	L.A. (mg/ml)			OD_{600nm}	Final pH	L.A. (mg/ml)
19.3	20°C	1.9 ± 0.1	4.6 ± 0.2	9.2 ± 0.4	**26.1**	20°C	5.5 ± 0.3	4.2 ± 0.3	
	28°C	1.9 ± 0.2	4.5 ± 0.2	10.6 ± 0.3		28°C	7.0 ± 0.1	3.9 ± 0.3	19.4 ± 0.4
	37°C	1.8 ± 0.1	4.7 ± 0.1	9.7 ± 0.1		37°C	4.5 ± 0.1	3.9 ± 0.1	
	NaCl 2%	1.4 ± 0.1	4.6 ± 0.1	8.5 ± 0.3		NaCl 5%	4.2 ± 0.3	3.9 ± 0.2	
	pH 5.5	1.2 ± 0.1	4.9 ± 0.1	8.5 ± 0.1		pH 4.5	5.1 ± 0.2	3.6 ± 0.3	12.5 ± 0.5
	BS 0.1%	0.8 ± 0.2	4.9 ± 0.2	3.2 ± 0.4		BS 0.1%	3.8 ± 0.2	4.1 ± 0.2	9.5 ± 0.4
P109	15°C	5.1 ± 0.5	4.9 ± 0.4	9.1 ± 0.3	**P124**	15°C	3.3 ± 0.4	4.6 ± 0.1	8.0 ± 0.2
	28°C	4.9 ± 0.1	4.3 ± 0.2	12.3 ± 0.1		28°C	3.8 ± 0.1	4.3 ± 0.3	12.3 ± 0.3
	37°C	3.0 ± 0.3	4.6 ± 0.3	12.3 ± 0.3		37°C	3.4 ± 0.2	4.5 ± 0.1	10.7 ± 0.1
	NaCl 5%	2.5 ± 0.1	4.4 ± 0.1	8.3 ± 0.1		NaCl 5%	2.0 ± 0.1	5.0 ± 0.2	9.1 ± 0.4
	pH 4.5	3.2 ± 0.4	4.1 ± 0.1	9.7 ± 0.3		pH 4.5	1.5 ± 0.1	4.0 ± 0.1	6.5 ± 0.4
	BS 0.1%	2.3 ± 0.3	5.4 ± 0.2	6.5 ± 0.4		BS 0.1%	1.1 ± 0.1	5.7 ± 0.2	5.6 ± 0.5
34.9	28°C	3.2 ± 0.2	4.5 ± 0.3	12.8 ± 0.1	**FV403**	20°C	2.9 ± 0.3	5.1 ± 0.2	17.0 ± 0.4
	37°C	4.3 ± 0.1	4.0 ± 0.2	18.1 ± 0.3		37°C	3.6 ± 0.1	4.4 ± 0.2	20.1 ± 0.1
	42°C	3.1 ± 0.1	4.1 ± 0.3	18.6 ± 0.4		42°C	1.5 ± 0.2	4.9 ± 0.3	20.0 ± 0.3
	NaCl 2%	3.4 ± 0.2	3.8 ± 0.2	13.6 ± 0.2		NaCl 5%	2.0 ± 0.1	4.6 ± 0.1	11.2 ± 0.4
	pH 4.5	2.1 ± 0.1	3.8 ± 0.2	13.8 ± 0.3		pH 4.5	2.8 ± 0.1	4 ± 0.1	16.4 ± 0.3
	BS 0.1%	1.2 ± 0.3	4.7 ± 0.1	3.7 ± 0.4		BS 0.1%	1.4 ± 0.5	4.6 ± 0.3	19.7 ± 0.6

The growth parameters measured after 24 h of incubation are presented in Table 3. All strains grew very well under their optimal conditions. Moreover, the higher and lower temperatures did not affect significantly the growth, OD and pH values being similar with the ones for optimal temperature. Strains 19.3, 26.1 and FV403 showed a very good growth in the presence of NaCl and in acidic growth medium, with OD values similar with the ones for optimal conditions. The two *Leuconostoc* strains and *L. helveticus* 34.9 were more sensitive to these stress conditions, the growth being slower than under optimal conditions.

The lowest OD values were recorded for the cultures obtained in the presence of 0.1% (w/v) bile salts. However, for almost all strains, these values were above 1. Lactic acid production was, in general, directly related with the cell growth, with the highest concentration for the control cultures and the lowest concentration for the cultures obtained in the presence of 0.1% of bile salts (Table 3).

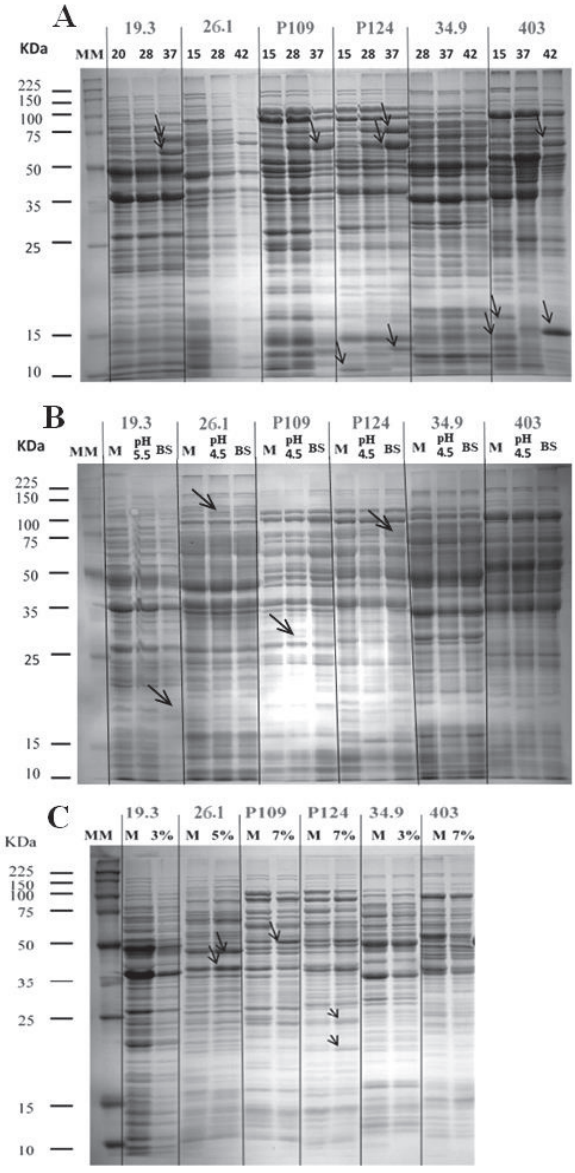

Figure 1. SDS-PAGE patterns of the total protein extracts from the cultures obtained at different temperatures (A), at low initial pH orin the presence of 0.1% BS (B), and in the presence of NaCl (C); MM - molecular weight marker; M - control cultures (optimal growth conditions)

SDS-PAGE analysis of protein profiles

SDS-PAGE of total proteins did not reveal significant differences for the bacterial cultures obtained at low initial pH or in the presence of NaCl or bile salts, except an over expression of few proteins for some strains (Figure 1). On the contrary, for bacterial cultures obtained at lower/higher temperatures, SDS-PAGE analysis showed several changes in the protein profiles. The most important one is the intensification of some bands of approximately 60-70 kDa, most probably corresponding to heat shock proteins (Figure 1). Moreover, intensification or disappearance of some bands of low molecular mass (10-20 kDa) was observed. Previous studies described various enzymes and proteins involved in cell protection (van de Guchte et al., 2002; Champomier-Verges et al., 2010) to stress. Chaperone proteins of approx. 60 kDa (dnaJ, dnaK), chaperonine Hs10 of 33kDa and chaperone GroS of 10 kDa are recognized as „stress proteins" and are observed as response to various stress factors as thermal stress, low pH, high pressure and osmotic stress (Champomier-Verges et al., 2010; Mills et al., 2011; Wu et al., 2012). Therefore, some bands that appeared more intense at high temperatures or in the presence of NaCl may correspond to such specific enzymes and proteins involved in cellular adaptation to stress.

Detection and measurements of enzymatic activities

LDH detection on SDS-PAGE gels was firstly made with both L-lactic acid and D, L - lactic acid (Figure 2). When L - lactic acid was used, bands of different intensities were observed only for *Lb. plantarum* 26.1 and *Lb. brevis* FV403. When D, L - lactic acid was used, other bands of different intensities appeared for both strains of *Leuc. mesenteroides*, most probably corresponding to D - LDH. Therefore, all the tests were further done with D, L - lactic acid. However, except the slight differences in the intensity of the bands, there were no other obvious changes in the electrophoretic patterns. Spectrophotometrical analysis did not show significant changes in the LDH activities. The activity of control cultures was, in general, slightly higher compared with the stressed cultures (results not shown).

Spectrophotometrically, MDH activity was observed only for two *Leuc. mesenteroides* strains, *Lb. helveticus* 34.9, and *Lb. brevis* FV403, but without significant differences between the growth conditions (results not

shown). On polyacrylamide gels (Figure 3), bands with different intensities could be observed for these strains, except for *Lb. helveticus* 34.9. However, the staining method does not seem to be very specific, since bands corresponding, most probably, with LDH, were detected for *Leuc. mesenteroides* P124 and *Lb. brevis* FV403.

Concerning the ADH activity, the analysis performed on protein extracts showed that the enzyme seems to be produced only by the two *Leuc. mesenteroides* strains and by *Lb. brevis* FV403. Generally, the ADH activity had similar values for all growth conditions, with slightly higher values for the cultures obtained under stress conditions, especially in the case of *Lb. brevis* FV403 grown at low initial pH or in the presence of bile salts (results not shown).

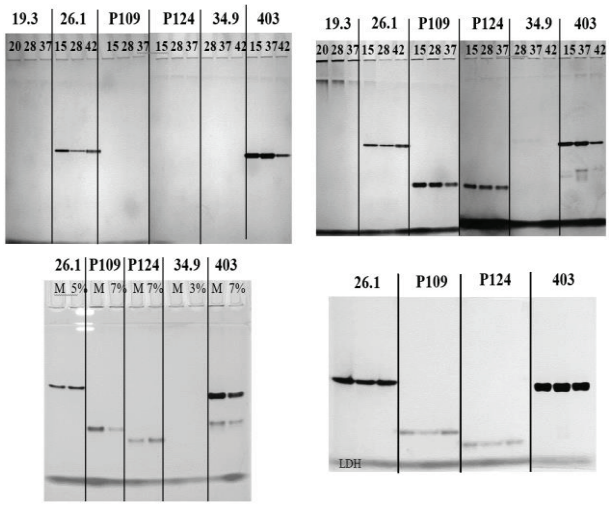

Figure 2. LDH patterns of the tested strains: first image - staining with L - lactic acid and the others - stained with D, L - lactic acid, M - optimal growth conditions

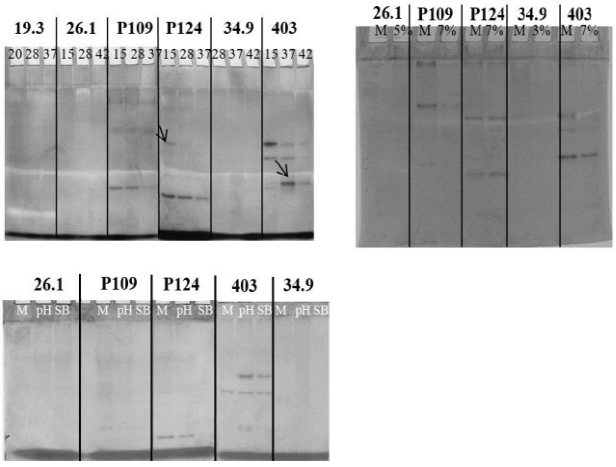

Figure 3. MDH patterns of the tested strains M - optimal growth conditions

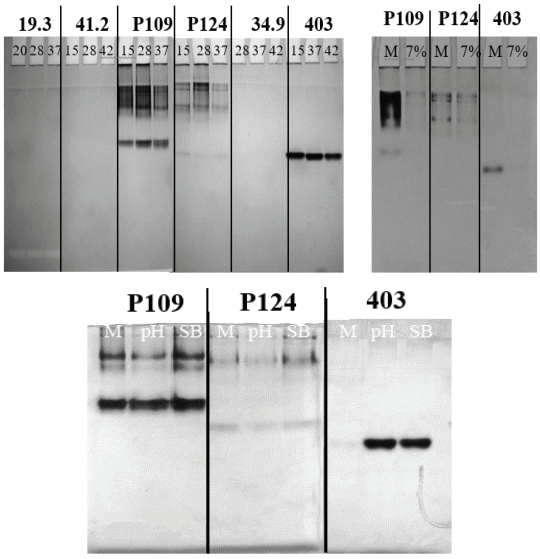

Figure 4. ADH patterns of the tested strains M - optimal growth conditions

The SOD activity was detected and measured only for *Lact. lactis* 19.3. For all stress conditions, higher values have been recorded compared with the control (Table 4). On the electrophoresis gels, a large variety of bands was observed, including in the protein extracts from other strains, proving a low specificity of the coloring method (results not shown).SODs belong to a group of antioxidant enzymes and have been shown to be involved in the protection against oxidative stress in various organisms, including LAB (Bruno-Bárcena et al., 2004), but we have also shown its potential involvement in other types of stresses, such as high or low incubation temperatures (Zamfir and Grosu-Tudor, 2014).

EPS isolation and quantification

Leuconostoc mesenteroides strains cultivated under optimal and stress conditions produced variable EPS quantities (Figure 5). *Leuc. mesenteroides* P109 was able to produce important quantities of EPS when cultivated both in optimal medium and stress conditions. As expected, the highest amount of EPS was recorded in the optimal growth conditions. The most important feature of this strain is the ability to produce EPS in presence of NaCl which can make it suitable for use in food industry (i.e. cheese industry), for texture improvement. Also, the ability of strain P109 to grow well and produce EPS in the presence of bile salts (0.1%), at low pH, and at the body temperature can recommend it as a functional strain. On the contrary, strain P124 recorded a

maximum EPS production in low temperature conditions (12.39 g/L). Synthesis of high quantities of EPS at low incubation temperatures can be explained as a defense mechanism of the cells against harsh environmental conditions. Both *Leuconostoc mesenteroides* strains showed a decrease of EPS synthesis when cultivated at a temperature higher than optimum.

Furthermore, for both *Leuc. mesenteroides* strains, the EPS synthesis in media with low pH or supplemented with 0.1% bile salts was correlated with the bacterial growth, namely lower than for optimal conditions.

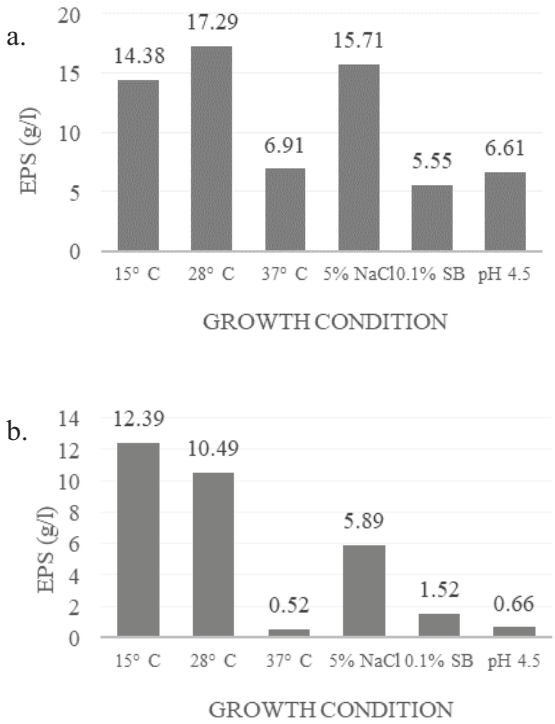

Figure 5. EPS production by *Leuconostoc mesenteroides* P109 (a) and P124 (b) under different growth conditions

Tabel 4. SOD activities of *Lact. lactis* 19.3 under different growth conditions

Sample		SOD (U/mg)
19.3	20°C	7.82
	28°C	2.8
	37°C	7.75
	NaCl 2%	6.5
	pH 5.5	3.62
	BS 0.1 %	4.02

Antibacterial activity

Lactococcus lactis 19.3 showed an important antibacterial activity against *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901^T (Table 5). The highest activity was observed for bacterial cultures obtained under optimal growth condition (25,600 AU/ml), but also at lower temperature (12,800 AU/ml). Moreover, this strain maintained a lower antimicrobial activity when grown in the presence of NaCl or in the acidic medium (3,200 AU/ml), but also in the presence of bile salts (200 AU/ml).

Lb. plantarum 26.1 showed the highest antimicrobial activity (800 AU/ml) against the indicator strain when grown under optimal conditions followed by cultivation at lower temperature (400 AU/ml) (Table 5). Also, a small activity of 200 AU/mL was observed in the medium with at low initial pH and in the presence of NaCl. For the other conditions, the antimicrobial activity ceased.

On the contrary, *Lb. helveticus* 34.9 had a small activity (200 AU/mL) in optimal conditions but eight times higher in the presence of NaCl (Table 5). Moreover, the highest antibacterial activity (3,200 AU/mL) of *Lb. helveticus* 34.9 was observed in the cultures obtained in the presence of bile salts.

Table 5. Antimicrobial activity of the bacteriocins producing strains in different growth conditions

		AU/mL
19.3	20°C	25,600
	28°C	25,600
	37°C	12,800
	2% NaCl	3,200
	0.1% BS	200
	pH 5.5	3,200
26.1	20°C	400
	28°C	800
	37°C	0
	5% NaCl	200
	0.1% SB	0
	4.5	200
34.9	28°C	0
	37°C	200
	42°C	100
	2% NaCl	1,600
	0.1% SB	3,200
	pH 4.5	200

This phenomenon has been previously described as in stress condition, the bacterial growth is slower, so the cells have more energy for the synthesis of bacteriocins. Moreover, higher concentrations of bacteriocins produced in stressful environments compensates for lactic acid deficiency, helping the bacterial cells to win in the competition for nutrients (Kanmani et al., 2012). The ability of *Lb. helveticus* 34.9 to produce more antimicrobial components when cultivated under stress conditions, especially in the presence of bile salts or NaCl can be an important feature of this strain. Firstly, it can be used as a functional strain because not only survives in the presence of BS, but also can act against other bacteria. Secondly, this characteristic of *Lb. helveticus* 34.9 to produce more antibacterial molecules under stress conditions can be exploited in food industry.

Isolation of S-layer proteins

Åvall-Jääskeläinen and Palva (2005) reported a molecular weight of the S-layer proteins produced by lactobacilli between 25 and 71 kDa. The SDS-PAGE electrophoresis of the extracellular proteins revealed two protein bands of 45-50 kDa, with a major one at about 47 kDa for *Lb. helveticus* 34.9 and a band corresponding to about 52 kDa for *Lb. brevis* FV 403 (Figure 6).

The electrophoretic profiles of S-layer proteins showed that *Lb. helveticus* 34.9 synthesizes more surface layer proteins when cultivated both under optimal conditions and increased temperature. Similar results have been reported by Frece et al. (2005), the authors suggesting that these proteins are preferentially expressed in conditions of thermal stress. On the contrary, in the presence of bile salts in the medium, the band corresponding to the molecular mass of S-layer is very thin. This can also be correlated with a weaker growth of the strain in this case. On the other hand, *Lb. brevis* FV403 seems to be able to produce more S-layer proteins at 20°C or in the presence of NaCl or in acidic environments. Therefore, the synthesis of S-layer proteins by *Lb. brevis* FV403 is more intense when stress factors are applied than under optimal conditions. Yet, no significant differences were observed in protein profiles of *Lb. brevis* FV 403 grown under different conditions.

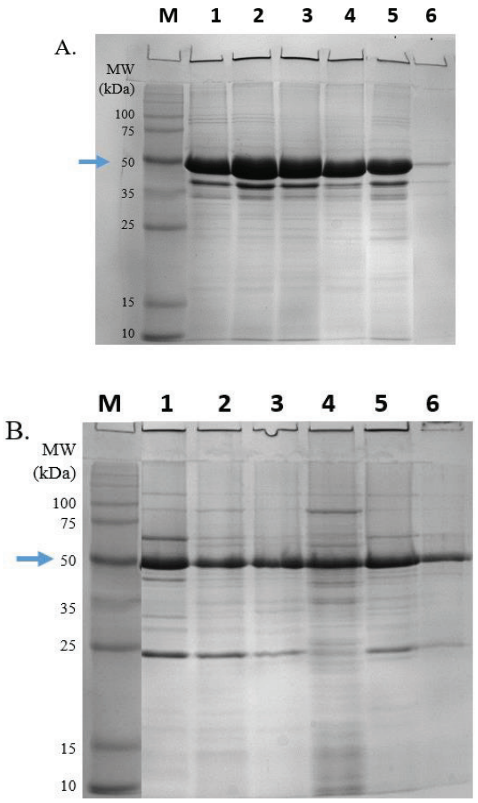

Figure 6. Electrophoretic profiles of S-layer proteins of Lb. helveticus 34.9 (A), and Lb. brevis FV403 (B) cultivated under different conditions:
A: 1 - 28°C; 2 - 37°C; 3 - 42°C; 4 - 2% NaCl; 5 - pH 4.5; 6 - 0.1% SB;
B: 1 - 20°C; 2 - 37°C; 3 - 42°C; 4 - 5% NaCl; 5 - pH 4.5; 6 - 0.1% SB; M - Molecular weight marke

CONCLUSIONS

Our study shows different responses of functional LAB to various types of stress. The most important finding is that all tested strains maintained their ability to produce important metabolites (i.e., bacteriocins, EPS, S-layer), more or less in all growth conditions, making them suitable for application in food industry, as functional starter cultures. In general, the synthesis of lactic acid, the enzymatic activities (except SOD activity), and biosynthesis of the bionanotechnologically important metabolites were directly related to growth, while under stress conditions, some modifications in the protein profile were observed. However, the bacteriocin production of *Lb. helveticus* 34.9 was enhanced by stressful environments (i.e. acidic media, bile salts addition) as a response or adaptation system to unfavorable conditions.

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