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Research Article

Molecular and in vitro assessment of some probiotic characteristics of amyolytic *Lactobacillus plantarum* strains from Bulgarian fermented products

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In the recent years, consumers' interest in healthy diet opened a new field for functional food development through combining the valuable composition of cereals and the health-promoting properties of lactic acid bacteria (LAB). LAB with amyolytic properties can assimilate starch in a single-step process and could be successfully applied as starter cultures offering an efficient nutritional conversion of cereal matrices. The probiotic potential of amyolytic LAB has not been investigated so far, therefore the present study focused on the molecular screening and in vitro tests of five amyolytic *Lactobacillus plantarum* strains to assess their tolerance to high acid and bile salts concentrations and antibiotic resistance as basic characteristics required for probiotic strains selection. Results showed excellent correspondence between the genetic screening and the phenotypic tests performed. Survivability at high acidity and bile salts presence was strain specific, with significant positive effect observed for cultures in stationary phase compared to those in exponential phase. Effect of starch in the medium proved most important to ensure viability of the amyolytic strains, which reveals the excellent potential of amyolytic LAB for commercially relevant probiotic applications. The strains proved to be generally safe in terms of antibiotic resistance. Among the five tested strains, *L. plantarum* Bom2 showed the best probiotic potential.

Keywords: Amyolytic lactic acid bacteria / In vitro assessment / *Lactobacillus plantarum* / Molecular screening / Probiotic properties

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1 Introduction

Amyolytic lactic acid bacteria (aLAB) are of high interest to science and food industry as they can be applied in fermentations of various starch-containing matrices for the production of foods with enhanced nutritional value, improved product safety and shelf life, and to bring health benefits to the consumer [1–4].

However, known aLAB strains are very few so far [5], and such strains with probiotic properties are not reported yet. Therefore selection of aLAB strains with probiotic characteristics is a perspective research target.

The benefits of probiotics include overall improvement of the digestive system activity by restoring and maintaining the balance of the intestinal microflora, suppressing the development of pathogenic and putrefactive microorganisms, modifying and eliminating toxins, lowering cholesterol levels, preventing diarrhea and urogenital infections, modulating the immune system by stimulation of the immune response, lowering body fat level, reducing stress and fatigue, regulating calcium-phosphorous exchange, preventing osteoporosis, etc. [6–11]. To ensure that such beneficial effects will be delivered by probiotics, a regular intake at least 10^6 – 10^7 viable cells is necessary [9, 12, 13].

Although the concept of probiotics is subject to many disputes and the European Food Safety Authority (EFSA) has so

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Abbreviations: aLAB, amyolytic lactic acid bacteria; EFSA, European Food Safety Authority; FAO, Food and Agriculture Organisation of the United Nations; FDA, The US Food and Drug Administration; GIT, gastro-intestinal tract; LAB, lactic acid bacteria; QPS, qualified presumption of safety; WHO, World Health Organisation

far rejected health claims for probiotics, Food and Agriculture Organization of the United Nations (FAO), the World Health Organization (WHO), and broad groups in the scientific, medical, and industrial environments support and continue the extensive research into selection of probiotic microorganisms and on finding reliable markers to confirm the health benefits associated with probiotics [6, 14, 15]. The need for scientific developments in the field is also supported by the worldwide steadily growing consumers' demand and probiotic products market. Functional food consumption is seen as a major industry trend benefiting global probiotics market growth, and in 2015, probiotic market size was USD 36.6 billion in terms of ingredient sales. This trend is getting popular as consumers are increasingly becoming aware of the link between nutrition, diet, and health. As a result, in the past few years, probiotics sales in Europe are increasing by 6.6% annually, while the forecast is that the global probiotic market will exceed USD 64 billion by year 2023 [<https://www.prnewswire.com/news-releases/probiotics-market-size-to-exceed-usd-64-billion-by-2023-global-market-insights-inc-578769201.html>].

However, to ensure consumer's safety and to prevent misleading health claims, it is essential that the industry clarifies specifications for probiotics in foods. In 2002, a FAO/WHO Expert Panel published a report with criteria for the selection and evaluation of probiotic microorganisms, and in 2007, EFSA introduced the "Qualified Presumption of Safety" concept as a fast track approach aiming to allow strains with an established history and safety status (e.g. natural isolates from traditionally consumed foods with proven health effects or from the gastro-intestinal system of healthy individuals) to enter the market without extensive testing requirements [16, 28]. Species that are safe for human consumption are listed in EFSA reference documents [17].

Initial screening for probiotic microorganisms includes phenotypic and genotypic characterization of the strains and analysis of their plasmid stability [6]; safety studies on acquired antibiotic resistance, virulence, and toxicogenicity; as well as hemolytic potential analysis of metabolic activity and prebiotic absorption capacity, tolerance to high acidity and temperature, tolerance to the presence of bile salts, testing of adhesive properties, and production of antimicrobials for pathogen inhibition [8, 9, 18–28]. Each probiotic candidate strain must be extensively characterized according to the above criteria and the probiotic properties may not be assumed for other microorganisms even when they are closely related [19, 29].

The health effects of the action of probiotic microorganisms are predetermined by their ability to survive the aggressive conditions in the gastro-intestinal tract (GIT) of the host and to colonize the intestinal epithelium to build stable microbial communities with the resident microflora [9, 24, 26, 27]. The first survival barrier is the high acidity in the stomach (pH 2–3) [23]. The low pH affects the proton-driving force of bacterial cells, thereby obstructing membrane transport and energy supply. In addition, low pH may suppress the activity of acid-sensitive enzymes from important biochemical pathways [30]. Bile salts are synthesized in the liver by cholesterol and are separated from the bile in a conjugated form, from where they fall into the duodenum. Bile salts act as detergents, destroying lipids, and fatty acids in the bacterial cell membrane [31, 32].

Concentration of toxic bile salts in the human body varies between 0.1 and 0.5% [27, 31], therefore resistance to such bile salts levels is another important criterion for the selection of potential probiotic microorganisms [24].

There are a number of in vitro and in vivo methods to study the survival of a probiotic strain in the GIT. In vivo methods are complex and expensive to perform, especially in the early stages of screening probiotic microorganisms [22]. A typical in vitro study of acid tolerance and survival in the presence of bile salts involves cultivating strains at low pH and a defined concentration of bile salts over a period of time during which survival is monitored [22, 27]. Dynamic in vitro models using a food matrix and simulating physico-chemical conditions in the stomach and small intestine are much more reliable and informative, since they allow to track not only the impact of the aggressive conditions in GIT but also the effect of the accompanying food matrix. However, such complex models are very expensive and highly labor-intensive for adaptation and implementation [27, 32].

Considering the enormous number of lactic acid bacteria (LAB) in the human gut and their beneficial effects, LAB resistance to antibiotics was initially considered to be a positive feature. However, in the recent years it was found that LAB may receive and transmit genes for antibiotic resistance to other types of bacteria, including pathogens. In addition to transmitting genes for resistance, LAB may also acquire those from other bacteria to survive in an antibiotic agent environment. Resistance genes are located on mobile genetic elements, such as plasmids and transposons. Such genes have been found in species of the genus *Lactococcus* [9], *Lactobacillus* [8, 33, 34], and *Enterococcus* [35]. Despite that LAB have been awarded the status of "Generally Recognized As Safe" by the Food and Drug Administration (FDA) [36] and included in the Qualified Presumption of Safety scheme for safety assessment of probiotic microorganisms of EFSA, these new findings drew the attention of EFSA, the Center for Disease Control and Prevention and WHO [37, 38], and the assessment of antibiotic resistance of LAB has become particularly important. Therefore, the definition of a safe strain has expanded to include the absence of antibiotic resistance genes in LAB, given the alarming increase of resistance in pathogenic and commensal bacteria worldwide [39, 40].

The most commonly used methods to study the antibiotic resistance of LAB are traditional phenotypic techniques based on the study of bacterial growth in the presence of an antibiotic agent. These are the diffusion method with antibiotic disks, the microdirection method, the agar gradient method or the commercial *E*-test [41]. More detailed studies of the genetic basis for antibiotic resistance are performed by PCR-based methods, plasmid profiling, or microarrays [42].

The conventional in vitro phenotypic analyses of probiotic properties of LAB are time, labor, and resource consuming, especially when a large number of strains need to be tested. On the other hand, advances in our knowledge of the genetic diversity of LAB and the increasing number of sequenced LAB genomes allow the functional properties of LAB strains to be studied more efficiently at the molecular level [11, 43].

This research approach for probiotic selection has attracted much attention worldwide over the past 2–3 years and the knowledge and experience gained are limited so far [43]. Therefore,

the aim of the current study was to examine some basic probiotic properties (acid and bile resistance and antibiotic resistance) of amylolytic *Lactobacillus plantarum* strains originating from Bulgarian cereal-based fermented products at the molecular level and to confirm the expression of the genes by in vitro testing.

2 Materials and methods

2.1 Amylolytic strains, medium, and storage conditions

Five amylolytic *L. plantarum* strains (5.2, BB₂, Bom₂, Lin₂, and Pro) were previously isolated from Bulgarian cereal-based drink boza and confirmed as starch assimilating. The strains were maintained in MRS broth (Oxoid®, UK) at 4°C (subcultured once a month) and in 20% glycerol for long-term storage at –20°C.

2.2 Tolerance to high acidity

The amylolytic isolates *L. plantarum* were inoculated (1%) in MRS broth (MRS-glucose) and MRS-starch (MRS broth without beef extract and with 2% soluble potato starch instead of glucose) and incubated at 37°C until reaching exponential phase ($t = 5$ h, OD₆₀₀ ~ 1.0) and stationary phase (24 h). Biomass from the activated strains was washed twice and resuspended in sterile saline solution (0.85% w/v). Acidity of the cell suspensions was corrected by 5 M HCl to pH 2.4 for exponential phase cells and to pH 2.3 for stationary phase cells, respectively [23]. Bacterial suspensions were incubated for 1 h at 37°C at 10 rpm. Samples were taken every 20 min to estimate the viable cell count (cfu/mL) on MRS agar (Oxoid®, UK). The plates were incubated for 48 h at 37°C.

2.2.1 Statistical analysis

The total viable cell counts were transformed into log units (log cfu/mL) and presented as mean values of three replicates $\pm$ SD. Assessment of the effect of the low pH and the type of carbon source on the total viable counts was performed by Student's *t*-test ($\alpha = 0.05$; MATLAB 2010 software).

2.3 Tolerance to bile salts

The amylolytic *L. plantarum* strains were activated in MRS broth (Oxoid®, UK) at 37°C until exponential phase ($t = 5$ h, OD₆₀₀ ~ 1.0) and stationary phase (24 h) were reached. Cells in the respective phase were washed twice and resuspended in sterile saline solution (0.85% w/v). Bacterial suspensions (1%) were inoculated in MRS-glucose and MRS-starch media containing 0.5% bile salts. Control samples were inoculated in the respective culture media without bile salts. The strains were incubated at 37°C for 24 h, samples were taken at 1, 2, 3, 4, 5, and 24 h to enumerate viable cells [27].

2.3.1 Statistical analysis

The total viable cell counts were transformed into log units (log cfu/mL) and presented as percentage of dead cells in the respective culture medium with bile salts compared to control samples. In order to assess the effect of bile salts and the type of carbon source on the total viable counts, Student's *t*-test was performed ($\alpha = 0.05$) by using MATLAB 2010 software.

2.4 Antibiotic susceptibility of the strains

Amylolytic *L. plantarum* strains were tested for susceptibility toward 12 antimicrobial agents: penicillin G (10 IU), bacitracin (10 IU), vancomycin (30 µg), imipenem (10 µg), ampicillin (10 µg), tetracycline (30 µg), erythromycin (15 µg), chloramphenicol (30 µg), streptomycin (300 µg), gentamycin (10 µg), ciprofloxacin (5 µg), and rifampicin (5 µg), by the disk diffusion method (antibiotic disks, Himedia Laboratories, Mumbai, India), following the CLSI guidelines [44, 45] and the criteria established by CLSA and Charteris et al. [46].

2.5 DNA isolation

Isolation of DNA from the LAB cultures was performed by using commercial kit Gene Jet™ Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., Waltham, USA) according to the manufacturer's instructions.

2.6 Real-time SYBR Green PCR method

Real-time SYBR Green PCR methods of Turpin et al. [43] and Ouoba et al. [47] were adapted for the detection of genes involved in the survival of *L. plantarum* strains at low pH and in the presence of bile salts, as well as for genes coding resistance to four antibiotics. The genes, primers used, and annealing temperatures are presented in Table 1. Analyzes were performed in final volumes of 25 µl for reaction. The PCR mix contained 60 ng DNA of each strain, 12.5 µl 2× GreenStar Master Mix (Bioneer, Korea), and 2 µl of each 1 µM primers (Bioneer, Korea). Test conditions were the following: UNG for 2 min, initial denaturation at 95°C for 15 min, 40 cycles consisting of denaturation for 10 s at 95°C, and primers annealing and elongation for 40 s at the temperatures presented in Table 1. The real-time SYBR Green PCR analysis is complemented by a dissociation step in the following temperature mode: 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. DNA from *L. plantarum* WCFS₁ was used as a positive control, based on the sequenced genome of the strain [http://www.ncbi.nlm.nih.gov/genome/1108?genome_assembly_id=170517].

2.6.1 Analysis of the results of the real-time SYBR Green PCR

The real-time SYBR Green PCR test was performed in duplicate. Using the specialized software of Applied Biosystems, the

Table 1. Primers used for screening of genes coding some probiotic properties of amylolytic *Lactobacillus plantarum* strains

Gene	Main function	Product of the target gene expression	Primers			Reference
			Orientation	Sequences (5'-3')	Annealing temperature (°C) ^{a)}	
groEL	Survival at low pH	Heat shock protein 60	F R	TTCCATGGCKTCAGCRATCA GCTAAYCCWGTGTCGATTCG	59.0	[43]
clpL	Survival at low pH and high bile salts levels	ATP-ase	F R	GCTGCCTTYAAAACATCATCTGG AATACAATTTTGAARAACGCAGCTT	59.0	[43]
Ir1516		Suggested esterase	F R	TRACCACTYTCWCCATTCAACAA CCACTAGCRATGACYAATACKGGTT	59.0	[43]
bsh	Tolerance to high bile salts levels	Cholylglycine hydrolase	F R	ATTCCWTGGWTWYTGGGACA AAAAGCRGCTCTNACAAWCKAGA	57.0	[43]
VanA	Antibiotics resistance	Vancomycin	F R	AAC AAC TTA CGC GGC ACT AAA GTG CGA AAA ACC TTG C	56.0	[47]
TetS		Tetracycline	F R	TGG AAC GCC AGA GAG GTA TT ACA TAG ACA AGC CGT TGA CC	56.0	[47]
StrA		Streptomycin	F R	CTT GGT GAT AAC GGC AAT TC CCA ATC GCA GAT AGA AGG C	55.0	[47]
ErmA		Erythromycin		AAG CGG TAA AAC CCC TCT GAG TCA AAG CCT GTC GGA ATT GG	59.0	[47]

^{a)} Annealing temperatures established in the present study

critical level and the baseline for each series of experiments were determined. After analysis of the results, the average melting temperature $\pm$ SD was calculated for the PCR products of the amplification of each of the screened genes (data now shown).

3 Results and discussion

LAB must have certain functional and technological characteristics and meet certain safety criteria in order to be used as probiotics, in probiotic products or as starter cultures for the production of cereal functional foods. In the present study, five *L. plantarum* strains (5.2, BB₂, Bom₂, Lin₂, and Pro) were investigated by molecular screening and in vitro tests for their ability to survive high acidity and bile salts presence, and for antibiotic resistance. The strains originate from samples of Bulgarian cereal-based drink Boza and they were identified by molecular methods. The strains were selected based on their amylolytic activity [48] with the concept to combine the ability to convert starch into lactic acid with probiotic characteristics for further potential applications of these special starter cultures for cereal-based functional food development.

The beneficial effects of LAB and their functionality as probiotics are predetermined by their ability to retain a large number of viable cells while passing through the host's GIT [49]. LAB with probiotic properties should be resistant to the highly acidic environment in the stomach where the secreted hydrochloric acid and pepsin lower the pH to about 2–3 [27] in order to reach and colonize the small intestine where they are exposed to the lethal effect of bile salts at levels of 0.1–0.3% [23, 27].

3.1 Resistance to high acidity

In the present study, tolerance of amylolytic *L. plantarum* strains to high acidity was determined after 1 h incubation at 37°C at pH 2.4 for cells at exponential phase (5 h) and pH 2.3 for stationary phase cells (24 h). The cultures were grown in MRS with glucose and MRS-starch broth in order to assess both the effect of the growth phase and the carbon source on the survival of the aLAB strains at low pH identical to that of the human stomach.

The tested amylolytic strains demonstrated good survival capacity at low pH levels (Table 2). It was found that the survival of the cultures was strain specific and dependent on the growth

Table 2. Acidity tolerance of amylolytic *Lactobacillus plantarum* strains cultivated in MRS-glucose and MRS-starch media

Culture growth phase	Strain	MRS-glucose		MRS-starch	
		Initial cell counts [log cfu/mL]	Surviving cells after 1 h incubation at low pH [%]	Initial cell counts [log cfu/mL]	Surviving cells after 1 h incubation at low pH [%]
Exponential phase (pH 2.4)	5.2	8.55 ± 0.07	31.44	8.20 ± 0.10	44.46
	BB ₂	8.29 ± 0.05	18.69	8.18 ± 0.03	28.96
	Bom ₂	8.62 ± 0.10	27.80	8.14 ± 0.15	50.87
	Lin ₂	8.33 ± 0.31	0.00	8.28 ± 0.09	29.07
	Pro	8.37 ± 0.05	20.59	8.43 ± 0.04	24.82
Stationary phase (pH 2.3)	5.2	9.17 ± 0.26	33.38	9.15 ± 0.06	45.79
	BB ₂	9.40 ± 0.05	24.79	9.14 ± 0.10	35.78
	Bom ₂	9.33 ± 0.31	29.94	9.04 ± 0.40	51.13
	Lin ₂	9.32 ± 0.07	21.20	9.21 ± 0.12	30.74
	Pro	9.17 ± 0.15	22.17	9.34 ± 0.30	40.26

phase as well as the type of carbon source used in the medium for initial incubation of the isolates.

As expected, cultures in the exponential phase were more susceptible to low pH than those in stationary phase both when precultivated in MRS-glucose and MRS-starch broth. After 60 min incubation at pH 2.4, the cell counts of strain *L. plantarum* Lin₂ were below the limit of detection (1.95 log cfu/mL), while the other four exponential phase isolates precultivated in MRS-glucose showed relatively better results of 18.69–31.44%.

Survival capacity at low pH was significantly higher for all tested *L. plantarum* strains analyzed in stationary phase compared to cultures in exponential phase. However, it is important to note that survival rates of both exponential phase and stationary phase cultures were higher when strains were precultivated in MRS-starch broth compared to MRS with glucose. For some of the strains, the positive effect of starch as the carbon source in the cultivation medium resulted in 20–30% higher survival rates than those observed for MRS-glucose cultures. When precultivated in MRS-starch broth, strains *L. plantarum* Bom₂, *L. plantarum* 5.2, and *L. plantarum* Pro showed the highest survival rates of about 51, 45, and 40%, respectively. Furthermore, it was observed that all of the strains in the stationary phase remained viable after 1 h incubation at pH 2.3 at levels above 21% in MRS-glucose and above 30% in MRS-starch broth.

This could be explained by the amylolytic nature of the isolates and their metabolic characteristics of starch uptake. The tested aLAB strains were isolated from the cereal-based fermented drink Boza in which they have evolved for a long time, and according to Lebber et al. [50], adaptation to a particular food source

determines to the greatest extent strains' ability for survival in unfavorable conditions.

Numerous scientific publications demonstrate the capacity of *L. plantarum* strains to survive under stressful conditions. Kalui et al. [51] reported 18 strains of *L. plantarum* retaining viability after 3 h incubation at pH 2.5, with 10% of these strains not surviving pH 2.0. In a study by Sieladie et al. [27], the authors reported that of 114 *L. plantarum* isolates, 43 were resistant at pH 3.0 for 2 h. Upon reduction in pH to 2.0, 27 isolates survived at rates above 50%. Resistance of *L. plantarum* to low pH is determined by genes encoding functional proteins protecting the cell wall, DNA, and other proteins; also encoding enzymes, catalyzing metabolic pathways for transformation of amino acids, etc. [43,51].

The screening of the five amylolytic *L. plantarum* strains for genes encoding survivability at low pH (groEL) and both low pH- and bile resistance (clpL and Ir1516) showed the presence of at least one of these genes for each strain (Table 3). Strains *L. plantarum* Bom₂, *L. plantarum* 5.2, and *L. plantarum* Pro tested positive for all three genes, which could be related to the highest rates survivability of stationary-phase cultures of these three strains in MRS-starch.

3.2 Resistance to bile salts

The tolerance of amylolytic *L. plantarum* strains to bile salts was monitored for 24 h for cultures in exponential phase (5 h) and in stationary phase (24 h) in MRS-glucose and MRS-starch broth containing 0.5% bile salts. Results obtained clearly show that the

Table 3. Screening of amylolytic *Lactobacillus plantarum* strains for genes encoding resistance to high acidity and high bile salt concentrations

Strain	Genes encoding resistance to high acidity			Genes encoding resistance to high acidity and high bile salt concentrations		
	groEL	clpL	Ir 1516	bsh	clpL	Ir 1516
5.2	+	–	+	–	–	+
BB ₂	–	+	–	–	+	–
Bom ₂	+	+	+	–	+	+
Lin ₂	+	+	+	–	+	+
Pro	+	+	+	–	+	+

Figure 1. Bile salts tolerance of amyolytic *Lactobacillus plantarum* strains cultivated in MRS-glucose medium with 0.5% bile salts content. EXP, exponential phase culture; ST, stationary phase culture

presence of bile salts in the MRS medium with glucose is not a limiting factor for the development of the tested aLAB strains (Fig. 1). The strains show varying levels of resistance against bile salts, yet the percentage of dead cells is relatively low. After 24 h incubation cultures in exponential phase showed cell count reduction in the range of 12.34 to 17.81%, and for the stationary phase cultures cell count decreased by in the range 8.16–12.04%. Statistical analysis shows that the effect of growth phase on bile salts resistance is statistically significant ($p < 0.5$) and much more pronounced than its effect on the tolerance of the tested aLAB strains to high acidity. This finding has been reported by other authors as well and it could be explained by the more aggressive effect of the highly acidic media on bacterial cells [52].

Results in Fig. 1 show another interesting trend, which is particularly noticeable due to the increased incubation time of the strains (24 h). Most of the studies available in the literature, LAB strains cultivation time in the presence of bile salts is between 3 and 6 h [27]. In the present work, cultivation time was increased in order to explore the ability of the aLAB strains to adapt to the presence of bile salts in the environment, as suggested by other authors [25]. This property is confirmed in the present study. Analysis of the results shows no significant effect of bile salts on the viability of the aLAB strains until the second hour of incubation. This trend persists until the fifth hour of incubation, during which a slight increase in the percentage of dead cells is registered. This observation is especially noticeable in strain *L. plantarum* 5.2. Obviously, the strains manage to adapt to bile salts in the period between the 2nd and the fifth hour of incubation. At the 24th hour, the percentage of dead cells is significantly increased, but even the maximum cell count reduction observed for strain *L. plantarum* 5.2 in exponential phase reached only 22.13%, and the maximum for stationary phase cultures was registered as 15.8% for strain *L. plantarum* Bom2.

The highest level of survival in the presence of bile salts in MRS with glucose was observed for strains *L. plantarum* Lin2 and Pro, followed by Bom2, BB2, and 5.2. This is contrary to the resistance of the strains to high acidity, which confirms that there is no direct link between acid tolerance and tolerance to

Figure 2. Bile salts tolerance of amyolytic *Lactobacillus plantarum* strains cultivated in MRS-starch medium with 0.5% bile salts content. EXP, exponential phase culture; ST, stationary phase culture

bile salts of lactobacilli [53]. Results of this study showing high survival of *L. plantarum* strains in the presence of bile salts are fully comparable to those of other authors [24, 25, 27, 52, 53]. The results showing adaptability to bile salts are also consistent with earlier findings [25, 27].

Figure 2 shows the survival ability of the tested aLAB strains cultivated in MRS-starch medium with 0.5% bile salts. Analysis of the results shows that the starch in the nutrient medium has a significant influence ($p < 0.05$) on the viability of the analyzed isolates only at the 24th hour of the incubation. The calculated values of the percentage of cell reduction, whether tested in logarithmic or stationary phase, are lower than those obtained in the cultivation of strains in MRS with glucose. Obviously, the assimilation of complex polysaccharides into the environment unlocks mechanisms for survival of the strains in stress conditions.

When cultivated in starch-containing medium with 0.5% bile salts, the strains tested in the log phase had cell reduction from 13.34 to 23.14% at the 24th hour, while for the cultures in stationary phase cell reduction ranged from 9.13 to 16.09%. These observations clearly show the significant effect of the cultures growth phase on their bile salt resistance, which is particularly distinguished ($p < 0.5$) at the 2nd and the 24th hour of incubation. In MRS-starch, the lowest cell reduction levels were observed for strains *L. plantarum* Bom2, Lin2, and Pro, followed by strains 5.2 and BB2. Adaptation of the studied amyolytic strains to the presence of bile salts was also confirmed in the starch-containing medium.

In conclusion, we can summarize that the studied amyolytic strains *L. plantarum* show good survival ability in conditions of high acidity and presence of bile salts, which determines their potential as candidate probiotics.

3.3 Antibiotic susceptibility

L. plantarum is one of the species listed in EFSA reference documents as safe for human consumption with the condition to assess their antibiotic susceptibility [17]. In the present work, the

Table 4. Screening for antibiotic susceptibility of amylolytic *Lactobacillus plantarum* strains

Antibiotic, concentration	Mechanism of activity	Strain	Inhibition zone [mm ± SD]	Classification	Screening for genes coding antibiotic resistance
Penicillin G, 10 U	<i>Inhibitors of cell wall synthesis</i>	5.2	22 ± 2.65	S	
R ≤ 14		BB ₂	28 ± 4.16	S	NA
I – 15–16		Bom ₂	18.66 ± 1.16	S	
S ≥ 17		Lin ₂	16 ± 3.06	I	
		Pro	21 ± 2.31	S	
^{a)} Bacitracin, 10 U		5.2	8 ± 0.00	ND	
		BB ₂	16 ± 2.00	ND	NA
		Bom ₂	8 ± 1.16	ND	
		Lin ₂	8 ± 1.16	ND	
		Pro	9 ± 1.16	ND	
^{b)} Vancomycin, 30 µg		5.2	0.00 ± 0.00	R	+
R ≤ 14		BB ₂	0.00 ± 0.00	R	+
I – 15–16		Bom ₂	0.00 ± 0.00	R	+
S ≥ 17		Lin ₂	1 ± 0.58	R	+
		Pro	0.00 ± 0.00	R	+
Imipenem, 10 µg		5.2	33 ± 3.06	S	
R ≤ 14		BB ₂	35 ± 4.16	S	NA
I – 15–16		Bom ₂	19 ± 1.16	S	
S ≥ 17		Lin ₂	23 ± 1.16	S	
		Pro	18 ± 2.31	S	
Ampicillin, 10 µg		5.2	32 ± 3.22	S	
R ≤ 12		BB ₂	24 ± 0.00	S	NA
I – 13–15		Bom ₂	10 ± 0.00	R	
S ≥ 16		Lin ₂	10 ± 1.16	R	
		Pro	20 ± 4.00	S	
Tetracycline, 30 µg	<i>Inhibitors of protein synthesis</i>	5.2	18 ± 2.31	I	+
R ≤ 14		BB ₂	26 ± 1.16	S	–
I – 15–18		Bom ₂	11 ± 0.58	R	+
S ≥ 19		Lin ₂	12 ± 1.16	R	+
		Pro	14 ± 2.00	R	+
Erythromycin, 15 µg		5.2	25 ± 0.58	S	–
R ≤ 13		BB ₂	22 ± 2.00	S	–
I – 14–17		Bom ₂	14 ± 0.00	I	–
S ≥ 18		Lin ₂	20 ± 0.58	S	–
		Pro	18 ± 1.16	S	–
Chloramphenicol, 30 µg		5.2	26 ± 3.13	S	
R ≤ 12		BB ₂	20 ± 1.16	S	NA
I – 13–16		Bom ₂	18 ± 2.31	S	
S ≥ 17		Lin ₂	9 ± 1.16	R	
		Pro	20 ± 1.16	S	
Streptomycin, 300 µg		5.2	6 ± 4.04	R	+
R ≤ 11		BB ₂	8 ± 0.00	R	+
I – 12–14		Bom ₂	2 ± 0.00	R	+
S ≥ 15		Lin ₂	6 ± 0.00	R	+
		Pro	7 ± 1.16	R	+
Gentamycin, 10 µg		5.2	7 ± 0.58	R	
R ≤ 12		BB ₂	10 ± 0.0	R	NA
S ≥ 13		Bom ₂	5 ± 0.58	R	
		Lin ₂	6 ± 1.16	R	
		Pro	6 ± 0.00	R	
Rifampicin, 5 µg	<i>Inhibitors of nucleic acid synthesis</i>	5.2	14 ± 2.000	I	
R ≤ 12		BB ₂	19 ± 1.155	S	NA
I – 14–18		Bom ₂	10 ± 0.000	R	
S ≥ 19		Lin ₂	12 ± 2.000	R	
		Pro	10 ± 1.155	R	

(Continued)

Table 4. Continued.

Antibiotic, concentration	Mechanism of activity	Strain	Inhibition zone [mm ± SD]	Classification	Screening for genes coding antibiotic resistance
Ciprofloxacin, 300 µg		5.2	3 ± 0.577	R	
R ≤ 18		BB ₂	8 ± 0.000	R	NA
I – 16–20		Bom ₂	1 ± 0.577	R	
S ≥ 21		Lin ₂	0.00 ± 0.00	R	
		Pro	0.00 ± 0.00	R	

R, resistant; I, intermediate; S, susceptible (values in mm); NA, Not analyzed; ND, Not defined.

^{a)} Susceptibility criteria for bacitracin are not established yet.

^{b)} Susceptibility testing not required by EFSA.

antibiotic susceptibility of the amylolytic *L. plantarum* strains was studied against 12 broad-spectrum antibiotics commonly used in the medical practice. The test-antibiotics were selected based on their mechanism of action—inhibitors of the cell wall synthesis, inhibitors of protein synthesis, and inhibitors of nucleic acid synthesis. Results of the study are summarized in Table 4 based on the criteria of Charteris et al. [39] and CLSI document M31-A3 [44].

Results of our study indicated that none of the amylolytic *L. plantarum* strains originating from Bulgarian boza were totally susceptible to all antibiotics tested and multiple resistance was observed. Strains 5.2, BB₂, and Pro are mainly susceptible to inhibitors of the cell wall synthesis—imipenem, penicillin, and in part to ampicillin. The tested aLAB strains are also susceptible to the action of erythromycin and chloramphenicol, which suppress protein synthesis. Sensitivity to the above antibiotics is common in lactobacilli. The results for the susceptibility of the investigated aLAB to the cell wall and cell protein synthesis inhibitors are fully comparable to earlier data from Danielsen and Wind [33] and Coppola et al. [54].

The five *L. plantarum* strains are resistant to antibiotics that inhibit cell wall synthesis, such as vancomycin, and two of the strains—Bom₂ and Lin₂—are also resistant to ampicillin. Many studies demonstrate the resistance of *Lactobacillus* species to vancomycin [8, 33, 34]. Resistance to this antibiotic is due to the presence of D-alanine-D-lactate in the peptidylglucan layer of the cell wall instead of the dipeptide D-Ala-D-Ala, which is actually the target molecule for vancomycin. For this reason, many gram-positive bacteria are resistant to the action of this antibiotic [8]. Resistance to vancomycin is natural and not acquired due to gene transfer, therefore it is not a hazard [34].

With regard to protein synthesis inhibitors, all tested strains were resistant to streptomycin and gentamicin, whereas three of the isolates—Bom₂, Lin₂, and Pro—were resistant to tetracycline. Only strain *L. plantarum* Lin₂ was found to be resistant to chloramphenicol. LAB resistance to aminoglycoside antibiotics, such as gentamicin and streptomycin, is also characteristic and intrinsic to *Lactobacillus* spp. due to the absence of cytochromes and electron transport chains in LAB. These components affect membrane permeability and the entry of aminoglycoside antibiotics into the cell [18]. Bacterial resistance *L. plantarum* to tetracycline presents a particular problem. LAB resistance to this antibiotic agent is considered to be acquired and there is a possibility for its transfer to other bacterial species [55]. Therefore,

in this case, it is very important to study antibiotic resistance by molecular approaches in order to clarify the genetic basis and the mechanism of acquiring resistance.

Three of the tested amylolytic *L. plantarum* strains (Bom₂, Lin₂, and Pro) are resistant to nucleic acid synthesis inhibitors ciprofloxacin and rifampicin. Resistance of *Lactobacillus* species to nucleic acid synthesis inhibitors has also been observed by other authors [18, 39]. However, this resistance is also defined as natural and characteristic of lactobacilli.

Results obtained for the studied amylolytic isolates show multiple antibiotic resistance. Similar observations have been reported for LAB by other researchers [40, 56].

The aLAB strains were also subjected to molecular screening for the presence of genes encoding resistance to vancomycin, tetracycline, streptomycin, and erythromycin. Resistance to vancomycin, which is intrinsic for lactobacilli, was compliant with the presence in all of the strains of gene VanA (Table 4). Gene TetS was detected in all strains except for *L. plantarum* BB₂, which showed susceptibility to tetracycline in the phenotypic tests. Strain *L. plantarum* 5.2 was positive for the gene, with intermediate results from the phenotypic tests. Resistance to tetracycline is not intrinsic to lactobacilli, which means that the gene TetS could have been transferred to the tested strains.

All strains were resistant to streptomycin, which was in full compliance with the detection of gene StrA. Resistance to aminoglycoside antibiotics, such as streptomycin and gentamicin, is considered to be intrinsic in the *Lactobacillus* genus and is attributed to cell wall structure and membrane impermeability, and in particular to the absence of cytochrome-mediated electron transport, which mediates drug uptake [57]. The presence of resistance determinants, such as *aac(6')*-*aph(2'')* for gentamicin and *aphE* for streptomycin resistance, has been reported in *L. plantarum* [40] and *Bifidobacterium longum* [36].

All strains were negative for the presence of gene ErmA encoding resistance to erythromycin. This finding was confirmed phenotypically with the exception of strain *L. plantarum* Bom₂, which showed intermediate susceptibility.

Surveys of antibiotic resistance phenotypes and determinants have been conducted with LAB strains of European, African, and Asian origin and resistance genes have been found in all of them [47, 58]. Results from the present study showed excellent correspondence between the genetic screening and the phenotypic tests for antibiotic resistance of the studied

aLAB strains. Since antibiotic resistance is a mandatory step in the safety study of LAB strains for food applications, it is important to obtain reliable results by applying both molecular and phenotypic screening.

4 Concluding remarks

Results from the present study showed excellent correspondence between the genetic screening and the phenotypic tests performed with the studied amylolytic strains. High acidity was more aggressive toward the strains compared to bile salts presence. Survivability at high acidity and bile salts presence was strain specific, with strains showing significantly higher survivability rates in stationary phase compared to exponential phase cultures. Effect of starch in the medium proved most important to ensure viability of the amylolytic strains, which reveals the excellent potential of aLAB for commercially relevant probiotic applications. The studied strains proved to be generally safe in terms of antibiotic resistance, with potential concern regarding tetracycline resistance of three of the strains, since it is a result of horizontal gene transfer and could be transmitted to other microorganisms. Among the five tested strains, strain *L. plantarum* Bom2 showed the best probiotic potential. Further studies are necessary to reveal more valuable characteristics of the strains both as probiotics and in terms of technological applications.

Practical application

Amylolytic lactic acid bacteria are of high interest to food industry as they can be applied in fermentations of starch-containing matrices for the production of foods with enhanced nutritional value and health benefits. Application of defined starter cultures is the key to optimizing the fermentation conditions and improving food quality and safety. However, the use of strains with probiotic properties would enable food producers to offer products with additional functional benefits to meet the consumer demand for healthy diet. No probiotic aLAB strains have been reported yet, therefore the concept to combine amylolytic activity and probiotic characteristics opens a new direction in starter cultures research and development. The present study employed molecular screening for genes coding probiotic characteristics of amylolytic *Lactobacillus plantarum* strains as a quick tool for probiotic assessment, which was confirmed by in vitro tests. Results also showed a good potential for food applications of the strains studied.

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