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ANTIBACTERIAL ACTIVITY OF LACTOBACILLUS PLANTARUM STRAINS ISOLATED FROM BULGARIAN GREEN CHEESE

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Abstract: Today's consumers pay more attention to their health and the increasing interest in probiotics and functional foods leads to their global popularization. Lactic acid bacteria have a wide application in the food industry. They play an important role in food quality and bio-preservation processes. Searching for foods with high nutritional value and functional properties is a factor for promotion of the traditional fermented products.

The object of this study is a traditional Bulgarian Green Cheese, which is made from sheep milk and is characterized with different technique of production, unlike other similar products. The Green Cheese has specific green color due to the presence of moulds on the cheese's crust and its unique flavor and taste are a result of the properties of lactic acid microbiota and its potential beneficial effects.

The isolated six strains were identified as representatives of the genera *Lactobacillus* after an examination of their morphological and biochemical profiles. For more precise identification more discriminative methods as 16S rRNA sequence analysis were used and the isolates were determined as representatives of the species *Lactobacillus plantarum*. *In vitro* antimicrobial activity of the lactobacilli was estimated. Isolated strains possess a broad spectrum of activity against food-borne bacterial pathogens.

The presence of lactobacilli in the Green cheese adds value to the product's functional qualities. The broad spectrum of antimicrobial activity of the isolated lactic acid bacteria is a promising advantage, suggesting their potential applications in different food technologies as a bio-preservative agent.

INTRODUCTION

Fermentation as a method for preservation is a proven ancient technology. Fermentation process ensures longer shelf life of the food products and it is responsible for their microbiological safety, improves the taste, texture and beneficial effects to the consumer.

Worldwide, fermented foods become more popular because of their good sensory qualities and positive influence for the human organism. Fermentation process is also related to the consumption of different substrates, reduction of toxic compound, for example aflatoxins, and production of antimicrobial agents as lactic acid, bacteriocins, hydrogen peroxide, etc. (Elizabeth Caplice et al., 1999; Giorgio Giraffa, 2004).

Bio-preservation is based on the mechanism of carbohydrates oxidation, for derivation of end products (acids and alcohols), which limit the growth of different pathogens, and in the same time it serves as energy source for the beneficial microorganisms (A.H. Soomro et al., 2002).

Lactic acid bacteria (LAB) are considered for main producers of valuable substances by fermentation in the food industry. Lactic acid bacteria (LAB) are among the most important microorganisms in the food industry due to their ability to produce valuable substances. Their health and therapeutic properties are reported in many studies and their application as starter cultures is widespread in the production of food products as fermented milk, cheeses and sausages.

Non-starter lactic acid bacteria are dominant representatives in cheese ripening processes. They maintain the environmental conditions, necessary for the correct conduction of the process and ensures the qualities of the end product. LAB are responsible not only for the sensory qualities of the cheese, but also for the health properties of the product. The protective properties that non-starter LAB possess are expressed in providing microbiological stability of food, reducing the risk of foodborne pathogens' growth and strengthening the human immune system (Luca Settanni, Giancarlo Moschetti, 2010).

After crust formation, lactic acid bacterial enzymatic systems are activated in appropriate temperature and humidity conditions. The process is specific for different cheeses and it must be strictly controlled. The texture and the taste are obtained by the action of proteolytic enzyme systems of LAB in the process of amino acids assimilation. The enzymatic activity of lactic acid bacteria is usually directly related to their antimicrobial properties in the cheese ripening processes. Studies with different strains of *Lactococcus lactis subsp. lactis*, *Leuconostoc mesenteroides, Leuconostoc pseudomesenteroides, Lactobacillus paracasei* and *Lactobacillus plantarum* are provided to study the action of the enzyme systems they possess. *Lactococcus* species have been found to possess the strongest oxidative and proteolytic activity. Through the activity of various enzyme systems, LAB are able to synthesize antimicrobial substances naturally in the

course of their metabolic activity (Leticia González et al., 2010).

The use of heterofermentative lactobacilli in the ripening process reduces the risk of contamination with harmful microorganisms that impair the quality, durability and safety of the final product. The beneficial effects of *Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus paracasei* and *Lactobacillus plantarum* as natural bio-preservatives are reported to possess antimicrobial properties against enterobacteria, yeasts and moulds. Their mechanism of action is associated with production of lactic acid, acetic acid and hydrogen peroxide, which are toxic to the pathogens, but harmless for the lactobacilli (Marek Aljewicz, Grazyna Cichosz, 2015; Sabrina da Silva Sabo et al., 2014).

The presence of lactic acid bacteria is also found in many homemade cheeses, to which no starters are added (Tropcheva R., et al., 2011). Mesophilic lactobacilli such as *Lactobacillus brevis, Lactobacillus lactis* and *Lactobacillus curvatus* are found in the ripening process of semi-hard cheeses, proving that they synthesize the substances needed to form the sensory profile of the product in the final ripening hours (Amarela Terzic-Vidojevic et al., 2007).

This study aimed to characterize the microbiota of the Bulgarian Green cheese (Cherni Vit cheese) and to estimate the beneficial properties of the lactic acid microflora of the product.

MATERIALS AND METHODS

Identification of LAB strains

The classical phenotypic characteristics were studied according to established phenotypic criteria. The cell morphology of the strains was estimated and the strains were tested for Gram staining and catalase reactions, gas production and growth ability in MRS broth at 30°C, 37°C for 2 days. Fermentation profiles were determined by API CHL 50 system for biochemical identification of lactic acid bacteria. Molecular characterization, based on 16S rRNA sequence was applied. Genomic DNA was isolated by HiPurA™ Bacterial Genomic DNA Purification Kit (HiMedia Laboratories) in accordance with the manufacturer's instructions. Ready To GoTM PCR beads (Amersham, Biosciences) and the primer set fD1 and rD1 for 16S rDNA PCR were applied. Obtained 16S rRNA PCR products were used as a template for a standard sequencing procedure (Macrogen Inc.). The sequences were compared with the available nucleotide database from the NCBI GenBank. A similarity of >98% to the 16S rRNA sequences of the reference strains was used as a criterion for the identification.

In vitro antibacterial tests

All six *Lactobacillus plantarum* strains were screened for antibacterial activity against different pathogenic bacteria by agar-diffusion method. The

antibacterial tests were performed with four different LAB samples against each bacterial pathogen - exponential culture, acidic supernatant, neutralized supernatant and milk, fermented by every single *L. plantarum* strain. Six bacterial species: *Escherichia coli* ATCC 25922, *Enterobacter aerogenes* ATCC 13048, *Staphylococcus aureus* ATCC 6538, as well as *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* (Collection of Department of Biotechnology, Faculty of Biology, Sofia University- DB-SU) and *Pseudomonas putida* (DB-SU) were used as test-cultures in antibacterial assays. The cultivation was carried out at 37°C for 24 hours, under anaerobic conditions. After that the cell-free supernatants were extracted. The antibacterial effect was determined by the zones of inhibition of the test-microorganisms. Pathogenic bacteria were grown under optimal conditions - temperature regime and selective nutrient media. In addition, for each test micro-organism, control plates were made in which the pathogen was cultured under the same conditions without the addition of lactobacilli and cell-free supernatants. Control samples have shown that the test microorganisms develop well under these conditions, which is a prerequisite for the reliability of the inhibitory effect due to the activity of the lactic acid bacteria.

RESULTS AND DISCUSSION

Initial characterization of LAB isolated from Bulgarian Green Cheese

Standard microbiological techniques and selective medias were used for isolation of LAB from homemade Bulgarian Green cheese. The primary identification was accomplished by morphological, physiological and biochemical methods. In all samples was observed a predominance of Gram (+) bacteria with a phenotype of *Lactobacillus* species. Six LAB strains were isolated from the Green cheese and were called C3, C4, C5, C6, C7 and C10.

Morphological and physiological characterization of the newly isolated lactobacilli

The first stage of the identification is to obtain a pure culture from each isolate. This stage is carried out by conventional microbiological methods including microscopic and macroscopic purity control. Isolated LAB were originally characterized according to classical approaches based on a set of different morphological and physiological-biochemical analyzes. All strains were determined as Gram (+), catalase-negative, non-sporulating and grow well in microaerophilic to anaerobic conditions. Our studies showed that six LAB isolates belong to the group of mesophilic lactobacilli, with an optimal growth temperature of 37°C.

Biochemical profile of the isolates

A conventional identification method - API 50 CHL system, was applied to characterize the biochemical profiles of the isolates. The results of the 49 carbon sources utilization, obtained after 48 hours of cultivation, were processed by API LAB plus version 5.1, according to the system implementation instructions. The isolates were classified as *Lactobacillus plantarum* with 99,9% probability (Table 1).

When identifying lactobacilli, it is difficult to achieve 100% certainty in the determination of belonging to a species, as the individual representatives have very similar morphological, biochemical and genetic characteristics (Ayele Nigatu et al., 2001). Due to these identification difficulties, API tests on lactobacilli do not give fully reliable information about the species belonging to newly isolated strains, so it is necessary to apply additional highly discriminatory methods.

Table 1. Identification of the newly isolated lactobacilli by API 50 CHL system and 16S rDNA sequence analysis

Strain	Identified as:	Probability [%] according to to API LAB identification	Probability [%] based on the BLAST analysis of the sequences
C3	*Lactobacillus plantarum*	99,90%	99,90%
C4	*Lactobacillus plantarum*	99,90%	99,90%
C5	*Lactobacillus plantarum*	99,90%	100%
C6	*Lactobacillus plantarum*	99,90%	99,90%
C7	*Lactobacillus plantarum*	99,90%	100%
C10	*Lactobacillus plantarum*	99,90%	100%

16S rRNA sequencing analysis

Sequence analysis is a recognized standard in the taxonomic identification of bacteria and is successfully applied to members of the genus *Lactobacillus*. For this reason, the six strains were sequenced using the isolated total DNA as a matrix. This analysis confirms previous data from the initial identification with other methods. After a BLAST analysis with available sequences in GenBank the strain species were determined. As a result of 16S sequencing of the isolates, their affiliation to the species *Lactobacillus plantarum* (Table 1).

Lactobacillus plantarum is a predominant representative of cheeses with different sensory profiles, such as Cheddar cheese (Véronique Demers-Mathieu et al., 2016), Mozzarella cheese (Angela Guidone et al., 2016), Gauda cheese (Nam Su Oh et al., 2016), traditional cow, sheep and goat cheeses from different

geographic regions (Miriam Zago et al. 2011, Fabricio Luiz Tulini et al. 2013, M.A. Herreros et al., 2003).

Antibacterial activity of the isolated Lactobacillus plantarum strains

The expression of antimicrobial properties is one of the leading criteria for the selection of probiotic strains of microorganisms (Parisa Shokryazdan et al., 2014). The contamination of fermentation processes in the food industry and end products with pathogenic microorganisms is a serious risk factor for human health and the consumption of such products is becoming a global problem (Arie H. Havelaar et al., 2015). For this reason, the six *Lactobacillus plantarum* isolates were screened for their antimicrobial properties. The tests being carried out with reference test cultures representative of the most commonly reported food associated and other pathogens.

The first step in the determination of the antimicrobial activity of our new LAB isolates was the ability of the lactobacilli strains to acidify the medium by decreasing the pH value of the culture medium MRS broth after 24 hours of culturing at 37°C (Table 2). The reported pH values range between 4.5 and 5.3 in the end of the exponential phase of the culture's growth. The effect of acidification is due to the synthesis of organic acids, mainly lactic acid, which are typical end products of the lactic acid bacteria metabolism (Mohamed Ali Abdel-Rahman et al., 2013).

Screening for antibacterial activity was performed by the agar diffusion method using live exponential lactobacilli cultures, cell-free supernatants after 24-hour culturing (in two variants - acidic and neutralized supernatants) and fermented with each culture milk samples (Table 2).

The results show that the isolated *Lactobacillus plantarum* strains have a very strong inhibitory activity against Gram (+) bacteria - *Bacillus subtilis* and *Staphylococcus aureus* (Table 2 and Fig. 1), while against Gram (-) bacteria (*Pseudomonas sp., Escherichia coli, Enterobacter aerogenes*) the effect is weaker (Table 2., Fig. 2 and Fig. 3), which is most likely due to the extra layers and components in the cell wall of this group of microorganisms that do not allow antimicrobial substances to enter the pathogen cells (Avik Khan et al., 2015). Cases of lactic acid bacteria having an inhibitory effect on Gram (-) microorganisms have been reported, and the activity being mainly due to bacteriocin-like protein substances or biosensors and, more rarely, to acids (Ami Patel et al., 2013; Diaz De Rienzo MA et al., 2015). Significant differences in the activity of live cultures, fermented milks and cell-free supernatants were observed. The comparison between acidic and neutralized supernatants shows a higher activity in the area of the lower pH values.

Table 2. Antibacterial activity of the isolated lactobacilli strains

Lactobacillus plantarum strain			C3	C4	C5	C6	C7
Cell-free acidic supernatants' pH			4,56	5,29	4,82	4,8	4,92
Zone of inhibition; mm	*Escherichia coli*	LC	15	16,5	16	17,5	15,5
		AS	12,5	10	11	9,5	10
		NS	0	0	0	0	0
		M	11,5	12,5	11,5	10,5	10,5
	Enterobacter aerogenes	LC	29	20	23	30	30
		AS	0	0	0	0	0
		NS	0	0	0	0	0
		M	14	12	14	16	17
	Pseudomonas aeruginosa	LC	15	12	15	8	8
		AS	0	0	0	0	0
		NS	0	0	0	0	0
		M	0	0	0	0	0
	Pseudomonas putida	LC	20	17	17	35	35
		AS	0	0	0	0	35
		NS	0	0	0	20	15
		M	20	17	35	35	33
	Bacillus subtilis	LC	35*	35	35	35	35
		AS	35	35	35	35	35
		NS	35	35	35	35	35
		M	35	35	35	35	35
	Staphylococcus aureus	LC	35	35	35	35	35
		AS	35	35	35	35	35
		NS	35	35	35	35	35
		M	35	35	35	35	35

LC – live exponential L. plantarum strains culture; AS – acidic supernatant; NS – neutralized with 5M NaOH supernatant (pH 5,8-5,9); M – fermented milk
**Maximal zone of inhibition is determined to be 35 mm*

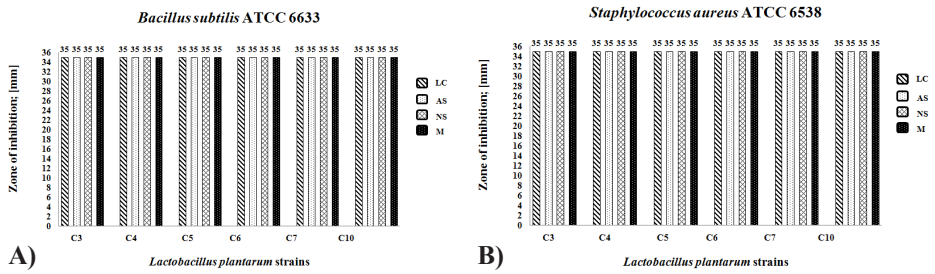

Figure 1. *Lactobacillus plantarum* strains' inhibitory avtivity against Gram (+) bacteria: A) *Bacillus subtilis* ATCC 6633; B) *Staphylococcus aureus* ATCC 6538

LC – live exponential L. plantarum strains culture; AS – acidic supernatant; NS – neutralized with 5M NaOH supernatant (pH 5,8-5,9); M – fermented milk

The lactobacilli strains studied in the present work are heterofermentative (Kandler O., 1983), i.e., along with lactic acid, they produce other organic acids such as acetic, benzoic and propionic acids. The organic acids with higher dissociation constant possess stronger inhibitory effect. Lactic and acetic acid refer to the group of weak lipophilic acids and their inhibitory effect is due to the undissociated molecules that predominate in the low pH medium (Abdellah Bouguettoucha et al., 2007).

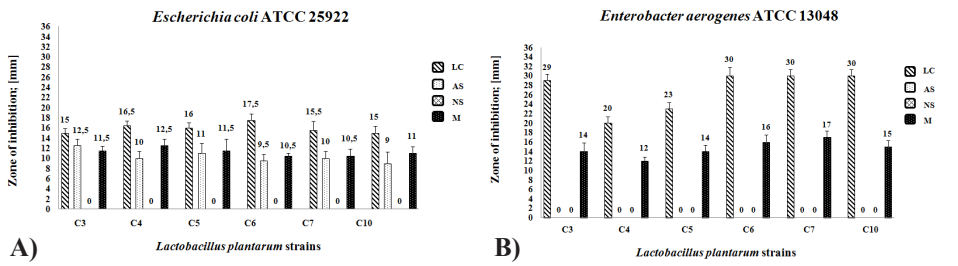

Figure 2. *Lactobacillus plantarum* strains' inhibitory activity against Gram (-) enterobacteria: A) *Escherichia coli* ATCC 25922; B) *Enterobacter aerogenes* ATCC 13048
LC – live exponential L. plantarum strains culture; AS – acidic supernatant; NS – neutralized with 5M NaOH supernatant (pH 5,8-5,9); M – fermented milk

The latter interact with major metabolic functions, such as substrate transport, oxidative phosphorylation, and proton release causing change in intracellular pH. All this destabilizes the membrane, which affects its permeability, at the same time leads to a decrease in the activity of a number of enzymes and can initiate the effect of other antimicrobial substances (Alakomi H.L. et al., 2000).

The presence of antimicrobial activity of the neutralized supernatants shows that the studied lactobacilli strains synthesize other inhibitory metabolites besides organic acids, which may be bacteriocin-like protein substances, hydrogen peroxide, short chain fatty acids, etc. In our study, the inhibitory activity of the newly isolated *Lactobacillus plantarum* against Gram (-) enterobacteria and *Pseudomonas* sp. representatives was observed (Fig. 3).

In the case of *Escherichia coli* and *Enterobacter aerogenes* no inhibitory effect was estimated with the neutralized cell-free supernatants. These results can be interpreted in terms of the pH optimum for the development of enterobacteria, which is in the neutral range, i.e., the inhibitory effect may be entirely due to the low pH of the medium maintained by the synthesized organic acids (Larissa B. Poppi et al., 2015). The strongest inhibitory effect demonstrated live-exponential cultures grown in MRS broth. The effect of the fermented milk samples is weaker (Fig. 2). The difference is due to the fact that lactobacilli produce different organic acids and other antimicrobial metabolites in MRS media and in milk (Jana Chramostova et al., 2014).

In the *Pseudomonas* sp. test, the inhibitory effect is mainly due to the organic acids synthesized in the medium (Fig. 3).

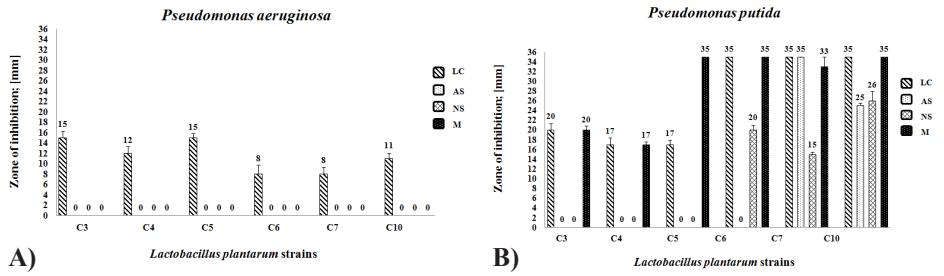

Figure 2. *Lactobacillus plantarum* strains' inhibitory avtivity against Gram (-) enterobacteria: A) Pseudomonas aeruginosa; B) Pseudomonas putida

LC – live exponential L. plantarum strains culture; AS – acidic supernatant; NS – neutralized with 5M NaOH supernatant (pH 5,8-5,9); M – fermented milk

From all of the testes Lactobacillus plantarum strains, the major bacterial growth suppression zones demonstrated strain C6. The general analysis of the antibacterial activity tests exhibits the presence of intra-species, strain variations in the activity of the isolates. The activity of the neutralized supernatants against Bacillus subtilis, Staphylococcus aureus and Pseudomonas putida provides a basis for further analysis in order to elucidate the nature of the inhibitory substances.

CONCLUSION

The results of the present study on the antimicrobial potential of the newly isolated *Lactobacillus plantarum* strains provide prerequisites for future studies related to the detailed analysis of the metabolites they produce in order to clarify their nature, mechanism of action and spectrum of antimicrobial activity. Such future analyzes would contribute to explaining these first promising results in more details at a fundamental scientific level and to provide a basis for a potential implementation of the new isolates as a therapeutic agent with antimicrobial properties or as biopreservatives in the food industry.

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Conflicts of Interest: The authors declare no conflict of interest.

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