I. MAN AND BIOSPHERE

GERANIUM SANGUINEUM L. - AN ALTERNATIVE SOURCE FOR ISOLATION OF LACTIC ACID BACTERIA

Tsvetanka Teneva, Dora Beshkova, Andrey Marchev, Milena Nikolova, Ginka Frengova, Atanas Pavlov

Abstract. In recent years it was observed a trend for increased interest in lactic acid bacteria (LAB), isolated from non-dairy environment due to their diverse metabolic profile and unique flavor-forming activities. Looking for new solutions to improve starter systems for healthy fermented foods and to expand opportunities for maximum utilization of biological potential of LAB, results in the idea of exploiting biodiversity of the unique natural bio-systems (medicinal plants). After large-scale screening of 300 microbial isolates (obtained from different parts of *Geranium sanguineum* L.) based on coagulation of milk, gas formation and non-specific odour and succeeding multiple transfer and growth in MRS and M17 selective media were isolated 169 single bacterial colonies. By differentiating tests were selected 62 Gram (+) and catalase (-) LAB. Of the representative lactic acid flora of *G. sanguineum* L. was studied acid-producing activity.

Keywords: *Geranium sanguineum* L., lactic acid bacteria, isolation, screening

INTRODUCTION

In recent years it was observed a trend for increased interest in lactic acid bacteria (LAB), isolated from non-dairy environment due to their diverse metabolic profile and unique flavor-forming activities. Looking for new solutions to improve starter systems for healthy fermented foods and to expand opportunities for maximum utilization of biological potential of LAB, results in the idea of exploiting biodiversity of the unique natural bio-systems (medicinal plants).

The medicinal plants are important ecosystem for isolation of LAB [1, 2]. Each particular plant species provides unique environment in terms of competitive microorganisms, natural plant antagonists, type, availability and concentration of substrate, and various physical factors. These conditions allow for the development of a characteristic epiphytic flora, bringing forth a population and sequence of fermentation microorganisms when the plant material is harvested and prepared for fermentation. It has been established that certain species of LAB are found in sequence, on various plants, indicating that they are natural habitats for lactobacteria, as the most frequently identified representatives belong to the genera *Leuconostoc, Lactobacillus, Pediococcus, Lactococcus, Streptococcus* [3, 4].

Currently available information for the use of medicinal plants as a source for isolation of LAB and their subsequent potential application as components for the formation of fermented milk starters is scarce. Plant-derived lactobacteria strains indicate tolerance to high pH value and salt concentration, ferment more types of carbohydrates and are much more resistant to stress as compared with those originating from milk; it wasn't marked any differences in the profiles of the enzymes, such as lipases, peptidases and phosphatases, necessary to obtain various fermented dairy products [5]. Michaylova et al. [6] first reported data of isolated strains of the species *Lactobacillus bulgaricus* and *Streptococcus thermophilus* from plants *Cornus mas*, *Prunus spinosa*, *Chrisanthemum*, *Dianthus* and others and proved that there are no significant differences in fermentation characteristics of plant-derived and commercial lactobacteria strains.

In the current work it is presented the possibility of using microbial diversity in the medicinal plant *Geranium sanguineum* L. for isolation of lactic acid bacteria with new metabolic activities, in order to include them as a starter component during in situ cultivation in milk.

MATERIALS AND METHODS

1. *Isolation and obtaining of bacterial isolates from G. sanguineum L.*

Plant material from *G. sanguineim* L., collected from three floristic regions: Eastern Rhodopes (Ivaylovgrad), Sofia region (the experimental field of the Institute of Botany, BAS) and Vitosha region (Iskar Dam) was aseptically processed and individual parts (flower, leaf and stem) were transferred in 12% skim milk (SM) (at 30°C and 37°C, until coagulation). Coagulated samples were cultivated in selective broth media- M17 (Merck, Darmstadt, Germany) and MRS (Merck, Darmstadt, Germany) with added cycloheximide (100 μg mL^{-1}) for 72h at 30°C and 37°C; subsequent growth of bacterial isolates on selective agarized media (Fig.1).

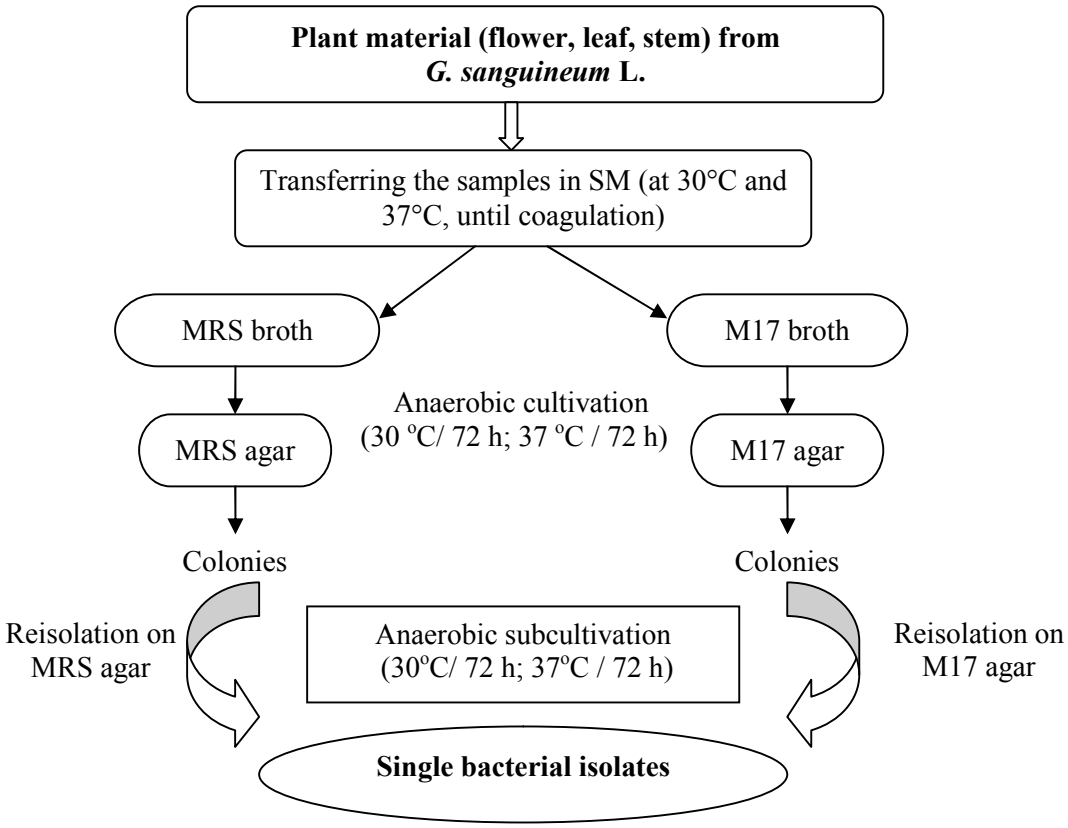

Fig. 1. Scheme for isolation of bacterial isolates from *G. sanguineum* L.

2. *Basic tests for identification of bacterial isolates*

Initial screening of the bacterial isolates included microscopic identification of morphology (shape, color and size) of the colonies (CETI, Digi Steddy II, Belgium), cell morphology (Micros Pink MC 50, Austria) and further differentiating Gram staining [7]. Biochemical tests (catalase test, [8] oxidase test [9, 10] indole reaction, [13] test for producing of CO_2 from glucose [11, 12]) were carried out of the selected bacterial isolates to be established their belonging to the group of lactic acid bacteria.

3. Acid-producing activity of suspected lactic acid (LA) isolates

The titratable acidity was established by titration of the samples with 0.1N NaOH, with phenolphthalein as indicator. Results were calculated as % lactic acid [13].

RESULTS AND DISCUSSION

In Figure 1 are presented the steps of obtaining pure bacterial isolates from available microbial flora on different parts of *Geranium sanguineum* L. After primary screening 300 microbial isolates (obtained from different parts of *Geranium sanguineum* L.) based on coagulation of milk, separation of gas and non-specific odour were selected 43 mixed isolates (14.3%). After succeeding multiple transfer and growth in selective MRS and M17 media were isolated 169 single bacterial colonies.

Obtaining LA isolates includes separate stages of phenotypic identification of pure bacterial isolates (Figure 2). Based on the results of used identification tests, out of 169 bacterial isolates, 62 showed phenotypic identity to the group of LAB (36.7%) (Table 1). From these suspected LA isolates 37 were isolated from flowers and 25 from leaves. There were no isolates from stems.

Morphological characteristic of representative colonies was determined after cultivation on selective agarized media (MRS and M17).

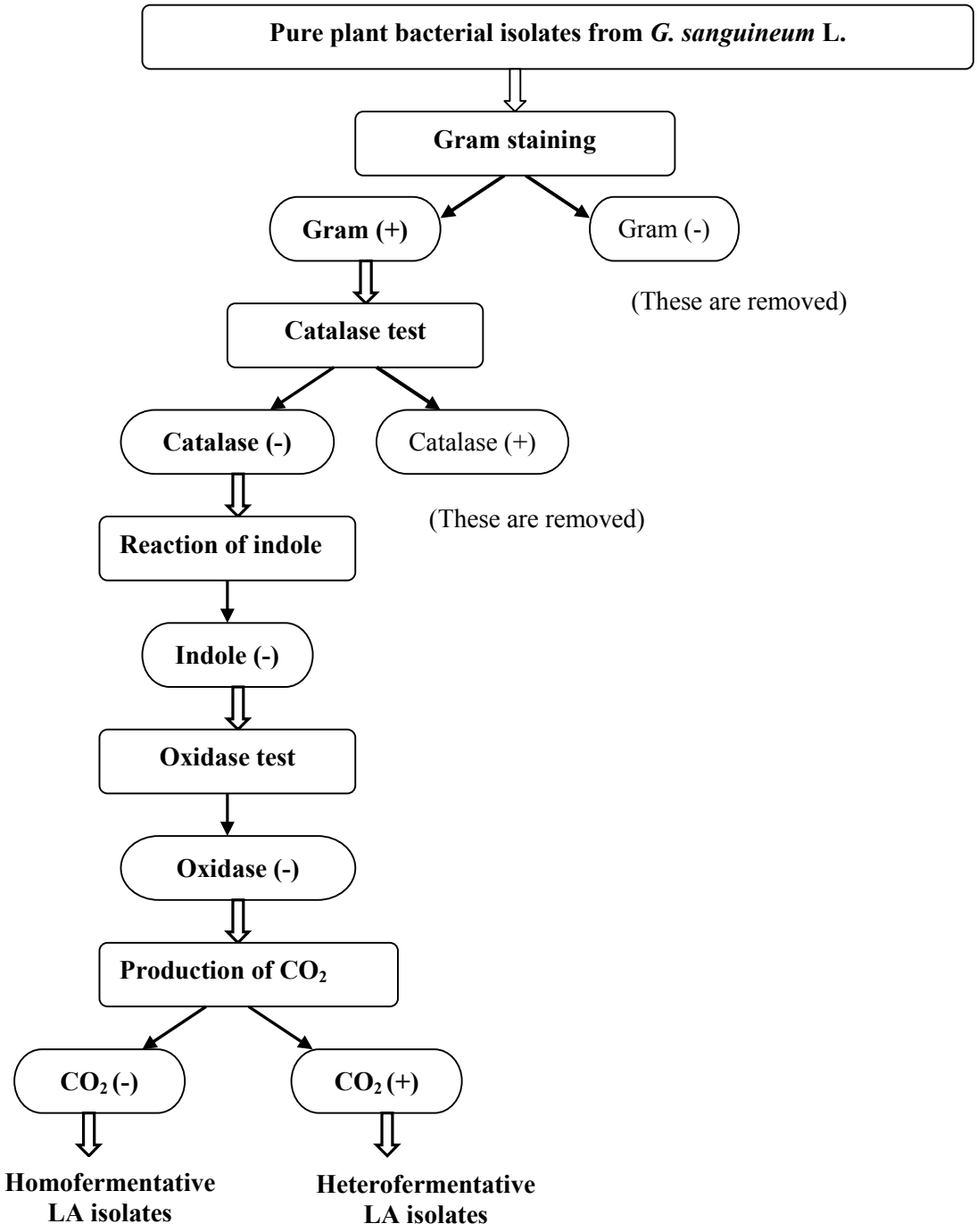

Fig. 2. Stages of phenotypic identification of LA isolates

A single colony was aseptically taken and transferred to the appropriate agar media and visually and microscopically differentiated. The cultural characteristics of the obtained single colonies are shown in Table 1.

All tests prove that the selected isolates belong to the group of LAB.

The data for acid- producing activity of LA isolates from *G. sanguineum* L. showed that with the highest producing ability of the examined isolates are those from the Iskar Dam, as 4 of them demonstrated extremely high activity (1.231 - 1.418% LA) compared to those from the Eastern Rhodopes and Sofia region (0.970% - 1.142 % and 0.792% -1.119%, respectively) (Table 2).

Table 1. Basic identification tests of LA isolates from *Geranium sanguineum* L. *

Bacterial isolates	Location	Morphology of	
		colony	cell
1	2	3	4
Gsf 2101	Sofia region	light beige, matt, round, margin convex and entire, 2205μm	Cocci, single, paired
Gsf 2115	Sofia region	light beige, matt, round, margin convex and entire, 1798μm	Cocci, single, paired
Gsf 2301	Sofia region	light beige, shinny, round, margin entire, 1284 μm	Cocci, short chain
Gsf 2302	Sofia region	light beige, shinny, round, margin convex and entire, 953μm	Cocci, short chain
Gsf 2303	Sofia region	light beige, shinny, round, margin convex and entire, 1057 μm	Cocci, short chain
Gsf 2304	Sofia region	light beige, shinny, round, margin entire, 1051 μm	Cocci, single, paired
Gsf 2305	Sofia region	light beige, shinny, round, margin entire, 841 μm	Cocci, paired, short chain
Gsf 2308	Sofia region	light beige, shinny, round, margin entire, 972 μm	Cocci, paired, short chain
Gsf 2310	Sofia region	light beige, shinny, round, margin entire,1156 μm	Cocci, paired, short chain
Gsf 2311	Sofia region	light beige, shinny, round, margin entire,1251 μm	Cocci, short chain
Gsf 2312	Sofia region	light beige, shinny, round, margin entire,760 μm	Cocci, short chain
Gsl 2201	Sofia region	light beige, shinny, round, margin entire,502μm	Cocci, single, paired, short chain
Gsl 2202	Sofia region	light beige, shinny, round, margin entire,515μm	Cocci, paired, short chain
Gsl 2212	Sofia region	light beige, matt, round, margin entire, 2009 μm	Cocci, single, paired
Gsl 2216	Sofia region	light beige, matt, round, margin entire,2050 μm	Cocci, single, paired
Gsl 2220	Sofia region	light beige, matt, round, margin entire,1988 μm	Cocci, single, paired
Gsl 2227	Sofia region	light beige, matt, round, margin entire,1580μm	Cocci, single, paired
Gsl 2228	Sofia region	light beige, matt, round, margin entire,1647μm	Cocci, single, paired
Gsl 2241	Sofia region	light beige, matt, round, margin entire,1542μm	Cocci, single, paired
Gsf 111	Eastern Rhodopes	white, shinny, round, margin entire, 1786 μm	Cocci, single, paired
Gsf 113	Eastern Rhodopes	white, shinny, round, margin entire, 1553 μm	Cocci, single, paired
Gsf 123	Eastern Rhodopes	white, shinny, round, margin entire, 1902 μm	Cocci, single, paired, short chain
Gsf 124	Eastern Rhodopes	white, shinny, round, margin entire, 1712 μm	Cocci, single, paired, short chain
Gsl 111	Eastern Rhodopes	white, shinny, round, margin entire, 2283 μm	Cocci, paired, short chain
Gsl 113	Eastern Rhodopes	white, shinny, round, margin entire, 1915 μm	Cocci, paired
Gsl 121	Eastern Rhodopes	white, shinny, round, margin entire, 1701 μm	Cocci, paired
Gsl 124	Eastern Rhodopes	white, shinny, round, margin entire, 1654 μm	Cocci, paired
Gsf 211	Eastern Rhodopes	light beige, shinny, round, margin entire, 538 μm	Cocci, single, paired
Gsf 221	Eastern Rhodopes	light beige, shinny, round, margin entire, 914 μm	Cocci, paired
Gsf 223	Eastern Rhodopes	light beige, shinny, round, margin entire, 869 μm	Cocci, paired
Gsl 212	Eastern Rhodopes	light beige, shinny, round, margin entire, 603 μm	Cocci, single, paired, short chain
Gsl 213	Eastern Rhodopes	light beige, shinny, round, margin entire, 647 μm	Cocci, single, paired, short chain
Gsl 222	Eastern Rhodopes	light beige, shinny, round, margin entire, 730 μm	Cocci, single, paired
Gsf 313	Eastern Rhodopes	light beige, shinny, round, margin entire, 1125 μm	Cocci, paired
Gsf 321	Eastern Rhodopes	light beige, shinny, round, margin entire, 963 μm	Cocci, paired

Continued to Table 1

1	2	3	4
Gsf 323	Eastern Rhodopes	light beige, shinny, round, margin entire, 928 µm	Cocci, paired
Gsl 311	Eastern Rhodopes	light beige, shinny, round, margin entire, 1052 µm	Cocci, paired, short chain
Gsl 312	Eastern Rhodopes	light beige, shinny, round, margin entire, 998 µm	Cocci, paired, short chain
Gsl 322	Eastern Rhodopes	light beige, shinny, round, margin entire, 732 µm	Cocci, paired, short chain
Gsf 51	Vitosha region	light beige, matt, round, margin undulate, 1435 µm	Cocci, paired, long chain
Gsf 52	Vitosha region	light beige, matt, round, margin undulate, 1374 µm	Cocci, paired, long chain
Gsf 53	Vitosha region	light beige, matt, round, margin entire, 1157 µm	Cocci, paired, long chain
Gsf 54	Vitosha region	light beige, matt, round, margin entire, 1122 µm	Cocci, paired, long chain
Gsf 60	Vitosha region	white, shinny, round, margin undulate, 1400 µm	Cocci, single, paired, long chain
Gsf 62	Vitosha region	white, shinny, round, margin undulate, 1583 µm	Cocci, single, paired, long chain
Gsf 64	Vitosha region	white, shinny, round, margin undulate, 1530 µm	Cocci, single, paired, long chain
Gsf 65	Vitosha region	white, shinny, round, margin undulate, 1470 µm	Cocci, single, paired, long chain
Gsf 67	Vitosha region	white, shinny, round, margin undulate, 1504 µm	Cocci, single, paired, long chain
Gsf 69	Vitosha region	white, shinny, round, margin undulate, 1439 µm	Cocci, single, paired, long chain
Gsf 71	Vitosha region	light beige, matt, round, margin undulate, 1574 µm	Cocci, paired, short chain
Gsf 72	Vitosha region	light beige, matt, round, margin undulate,1861 µm	Cocci, long chain
Gsf 74	Vitosha region	light beige, matt, round, margin undulate,2104 µm	Cocci, paired, short chain
Gsf 76	Vitosha region	light beige, matt, round, margin undulate,1954 µm	Cocci, paired, long chain
Gsf 77	Vitosha region	light beige, matt, round, margin undulate, 1245 µm	Cocci, single, paired, long chain
Gsf 78	Vitosha region	light beige, matt, round, margin undulate,1364 µm	Cocci, paired, short chain
Gsl 35	Vitosha region	light beige, shinny, round, margin convex and entire, 1502µm	Cocci, short chain
Gsl 36	Vitosha region	light beige, shinny, round, margin convex and entire, 1274µm	Cocci, short chain
Gsl 37	Vitosha region	light beige, matt, round, margin convex and entire, 873µm	Cocci, paired
Gsl 63	Vitosha region	light beige, matt, round, margin convex and entire, 1149µm	Cocci, short chain
Gsl 64	Vitosha region	light beige, matt, round, margin convex and entire, 687µm	Cocci, paired
Gsl 65	Vitosha region	light beige, shinny, round, margin convex and entire, 2032µm	Cocci, short chain
Gsl 66	Vitosha region	light beige, matt, round, margin convex and entire, 1978µm	Cocci, paired

* All the isolates are Gram (+), catalase (-), oxidase (-), indole (-) and not producing CO_2 from glucose.

The data for acid- producing activity of LA isolates from *G. sanguineum* L. showed that with the highest producing ability of the examined isolates are those from the Iskar Dam, as 4 of them demonstrated extremely high activity (1.231 - 1.418% LA) compared to those from the Eastern Rhodopes and Sofia region (0.970% - 1.142 % and 0.792% -1.119%, respectively) (Table 2).

Table 2. Acid- producing activity of LA isolates from *G. sanguineum* L.

LA isolates	TA (% lactic acid)	pH	LA isolates	TA (% lactic acid)	pH
Gsf 2101	0,985±0,045	4,55	Gsl 213	1,098±0,026	4,51
Gsf 2115	1,097±0,022	4,52	Gsl 222	1,052±0,022	4,51
Gsf 2301	0,803±0,011	5,01	Gsf 313	1,142±0,022	4,50
Gsf 2302	0,815±0,022	5,00	Gsf 321	1,007±0,022	4,51
Gsf 2303	0,792±0,005	5,03	Gsf 323	0,970±0,018	4,53
Gsf 2304	0,792±0,005	5,04	Gsl 311	1,097±0,022	4,50
Gsf 2305	0,805±0,011	4,98	Gsl 312	0,970±0,018	4,53
Gsf 2308	0,822±0,026	4,99	Gsl 322	0,985±0,004	4,53
Gsf 2310	0,810±0,011	5,00	Gsf 51	1,313±0,018	4,37
Gsf 2311	0,820±0,022	4,98	Gsf 52	1,418±0,018	4,35
Gsf 2312	0,792±0,005	5,04	Gsf 53	1,298±0,045	4,40
Gsl 2201	0,963±0,022	4,55	Gsf 54	1,231±0,022	4,40
Gsl 2202	1,119±0,004	4,52	Gsf 60	0,918±0,022	4,54
Gsl 2212	1,052±0,022	4,53	Gsf 62	0,857±0,022	4,59
Gsl 2216	1,097±0,022	4,50	Gsf 64	0,896±0,005	4,56
Gsl 2220	0,918±0,022	4,58	Gsf 65	0,940±0,005	4,53
Gsl 2227	0,985±0,004	4,57	Gsf 67	1,007±0,022	4,51
Gsl 2228	0,985±0,005	4,55	Gsf 69	1,052±0,022	4,50
Gsl 2241	0,940±0,005	4,56	Gsf 71	1,000±0,018	4,52
Gsf 111	1,097±0,022	4,52	Gsf 72	0,985±0,004	4,53
Gsf 113	1,097±0,022	4,51	Gsf 74	0,918±0,022	4,54
Gsf 123	1,122±0,004	4,50	Gsf 76	0,963±0,022	4,54
Gsf 124	1,072±0,029	4,52	Gsf 77	0,985±0,005	4,54
Gsl 111	1,142±0,022	4,49	Gsf 78	1,015±0,018	4,51
Gsl 113	0,985±0,005	4,55	Gsl 35	0,788±0,011	5,10
Gsl 121	1,000±0,018	4,53	Gsl 36	0,782±0,004	5,10
Gsl 124	1,142±0,022	4,50	Gsl 37	0,792±0,011	5,06
Gsf 211	1,009±0,026	4,51	Gsl 63	0,776±0,016	5,15
Gsf 221	0,958±0,017	4,55	Gsl 64	0,776±0,022	5,13
Gsf 223	1,075±0,005	4,52	Gsl 65	0,788±0,005	5,09
Gsl 212	1,119±0,006	4,51	Gsl 66	0,784±0,011	5,10

The results obtained are comparable with data reported by other authors who isolated, lactic acid bacteria from plants (0.85-1.10% LA) [6] and from sourdough (0.97-1.93% LA) [14]. The acid-producing activity of plant-derived examined isolates is close to those available in the lactobacteria collection of the research team (*Lactococcus lactis* ssp. *lactis* biovar *diacetylactis*- 1.16%, *Lactococcus lactis*- 1.39%, *Lactococcus cremoris*- 1.12%, *Streptococcus thermophilus*- 1.30%, *Enterococcus faecium*- 1.21%), isolated from dairy products.

In conclusion, it can be resumed that the selected phenotypic and biochemical characterized alleged LA isolates of medicinal plant *G. sanguineum* L. are potential representatives to be included as a component in starter communities during in situ cultivation in milk.

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GERANIUM SANGUINEUM L. – АЛТЕРНАТИВЕН ИЗТОЧНИК ЗА ИЗОЛИРАНЕ НА МЛЕЧНОКИСЕЛИ БАКТЕРИИ

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Резюме. През последните години се наблюдава тенденция за повишен интерес към млечнокисели бактерии (МКБ), изолирани от не-млечна среда, поради разнообразния им метаболитен профил и уникалните вкусово-формиращи активности. Търсенето на нови решения за усъвършенстване на стартерните системи за здравословни ферментирали храни и за разширяване на възможностите за най-пълно използване на биологичния потенциал на МКБ, доведе до идеята за експлоатиране на биоразнообразието в уникални природни биосистеми (лечебни растения). След широкомащабен скрининг на 300 микробни изолата (получени от различни части на *Geranium sanguineum* L.) на база коагулация на мляко, отделяне на газ и неспецифичен аромат и последващ многократен трансфер и развитие в селективни MRS и M17 среди, бяха изолирани 169 единични бактерийни колонии. Чрез диференциращи тестове бяха селектирани 62 Gram (+) и каталазо (-) МКБ. На представителната млечнокисела флора на *G. sanguineum* L. беше изследвана киселинопродуцираща активност.

Ключови думи: *Geranium sanguineum* L., млечнокисели бактерии, изолиране, скрининг

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