Scientific Bulletin. Series F. Biotechnologies, Vol. XIX, 2015
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

RESISTANCE PROFILE OF PLANT-DERIVED LACTIC ACID BACTERIA AGAINST HERB EXTRACTS

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Abstract

It was examined the possibility for growth of representatives from genera Enterococcus and Streptococcus, isolated from different parts of the herbs Salvia and Geranium in the presence of relevant crude plant extracts. There have been established relationships between the type and concentration of the herbal extracts and tested microorganisms. It was determined a resistance profile of examined lactic acid bacteria against the effect of natural plant extracts. It was established that Streptococcus thermophilus and Enterococcus faecium (isolated from Geranium) grow in the presence of extract of Geranium sanguineum L. in concentrations up to 30 mg/ml whereas some strains S. thermophilus and Ent. faecium (isolated from Salvia) show higher resistance - in range of 30 mg/ml to 100 mg/ml extract of Salvia officinalis L. This is an evidence of selective inhibitory activity of plant extracts to examined lactic acid bacteria and it is a prerequisite for their incorporation in food fermentation that have a lot of economic value, as well as in helping improve human health.

Key words*: lactic acid bacteria, plant extract, resistance profile, Salvia, Geranium.*

INTRODUCTION

In recent years there is a trend of increased interest in lactic acid bacteria (LAB), isolated from non-dairy products due to their diverse metabolic profile and unique flavor-forming activities. Plant – derived lactobacteria, have shown tolerance to high values of pH and salt concentration, high level of stress resistance and an ability to ferment more types of carbohydrates, compared to those of dairy origin. Furthermore, no significant differences were noted in fermentation characteristics and profiles of enzymes (lipases, peptidases and phosphatases) required for obtaining various fermented dairy products with plant and commercial strains lactobacteria (Nomura et al., 2006; Michaylova et al., 2007; Siezen et al., 2008; Venugopalan et al., 2010). Medicinal plants are an important ecosystem for isolation of LAB (Siezen et al., 2008; Venugopalan et al., 2010; Cakir, 2010; Baradaran et al., 2012). Each specified plant species provides a unique environment in terms of competing microorganisms, natural plant antagonists, as well as accessibility, type and concentration of the substrate in the various physical factors. These conditions allow growth of typical epiphytic flora, from which derives a population and a chain of fermentation processes, when the plant material is collected and prepared for fermentation.

Recently, it has been formed a new direction in scientific research, related to: obtaining bioactive or biogenic substances, extracted from different plants or synthesized, during food fermentation; subsequent creation of novel foods (defined as healthy and functional) by additional introduction into their technological schemes of such exogenous functional components or use of microorganisms, producers of biogenic substances, as well as microorganisms, which have probiotic characteristics (Gobbetti et al., 2010).

The genus *Salvia* is a broad genus belonging to the family Lamiaceae. *Salvia* comprises one of the largest genera of flowering plants in the world with 900 to 950 species occurring worldwide except in Australia (Ali and Aboud, 2010). Different species of *Salvia* genus are grown and used as a spice and in traditional and folk medicine, because of their antibacterial,

antioxidant, anti-inflammatory and analgesic properties (Ibrahim, 2012). Phytochemical studies conducted on plants of this genus have led to the isolation of diterpenoids (Habibi et al., 2000; Nieto et al., 2000), triterpenes, sterols (Rauter et al, 2007), anthocyanins, coumarins, polysaccharides, flavonoids and phenolic acids (Lu and Foo, 2002). *Salvia officinalis* (common sage) is known as one of the herbs that has antimicrobial activity, it is a useful resource in combating many illnesses. (Eidi et al., 2006; Ali and Aboud, 2010). It is cultivated in several countries mainly to be used in medicine, perfumery and food industry (Santos et al, 2002).

Geranium sanguineum L., commonly called Bloody Cranesbill, is an herbaceous plant species in the Geraniaceae family. It is native from Europe and temperate Asia (Hammami et al., 2011). It is wide-spread in Bulgaria and known with the popular name bloody geranium. Extracts from various parts of *G. sanguineum* L., have significant antiviral, antibacterial, anti-inflammatory and antioxidant activities (Serkedjieva and Manolova, 1992; Hammami et al., 2011). The *Geranium* genus phytochemistry is well known in present with the most studied classes of active compounds (tannins, volatile oils, flavonoids and polyphenols) (Fodorea, et al., 2004; Kobakhidze and Alaniya, 2004).

The aim of this study was to evaluate resistant profile of LAB isolated from medicinal plants *G. sanguineum* and *Salvia* species against extracts from plants from which they are isolated and to compare them to LAB isolated from dairy products; to select resistant LAB suitable for their potential cultivation in the presence of plant extract during *in situ* cultivation in milk.

MATERIALS AND METHODS

Microorganisms

Thirty one strains *Streptococcus thermophilus* and *Enterococcus faecium* used in this study were isolated from medicinal plants *Geranium sanguineum* L. (Teneva et al., 2014) and various *Salvia* species (*S. scabiosifolia* Lam., *S. ringens* Sibth. & Sm., *S. officinalis* L. and *S. blepharophylla* Brandegee ex Epling), collected from their natural habitats in Bulgaria as well as the Botanical garden of the Technical University in Dresden, Germany. LAB were grown in M17 broth (pH 6.6) (Merck, Germany, Darmstadt) at 37 ºC for 48 h.

Plant Materials and Preparation of crude extract

Plant materials of *Salvia officinalis* L. (young leaf) and *Geranium sanguineum* L. (root) were collected in June 2013 from their natural habitat - field near by town of Kroumovgrad and Vitosha region (Iskar Dam), respectively. The plant materials were washed 2-3 times with running tap water and once with sterile distilled water, air-dried, powdered and used for extraction.

100 g powder of each plant materials were extracted (in triplicate) with 70 % ethanol (1:10 w/v) for 24 h at room temperature. The crude extracts were pooled, filtrated by filter paper and concentrated in a vacuum evaporator and then they were lyophilized. Lyophilized extracts were dissolved in 30% ethanol and in water and subsequently filtrated through a 0.22 μm syringe filter.

HPLC analyses

The phenolic acid, flavonoids and quercetin glycosides were analyzed by HPLC system consisting of Waters 1525 Binary Pump (Waters, Milford, MA, USA), Waters 2487 Dual λ Absorbance Detector (Waters, Milford, MA, USA), controlled by Breeze 3.30 software. Supelco Discovery HS C18 column (5 μm, 25 cm × 4.6 mm) operated at 26 °C was used for separation. The following mobile phases were used for separation: of the phenolic acids - 2% (v/v) acetic acid (solvent A) and 0.5 % (v/v) acetic acid : acetonitrile (1:1, v/v) (solvent B); of the flavonoids - 2.0 % (v/v) acetic acid (solvent A) and methanol (solvent B), of the quercetin glycosides - 2.0 % (v/v) acetic acid (solvent A) and acetonitrile (solvent B). The gradient programs used for the phenolic acids and the flavonoids was described by Marchev et al. (2011) and for the quercetin glycosides by Ivanov et al. (2014). Eluting compounds were detected by monitoring the eluate at 280, 380 and 370 nm, respectively.

Rosmarinic acid was analyzed by the same Waters HPLC system and column. It was used

an isocritic elution with the following composition of the mobile phase and conditions: methanol : k. H_3PO_4 (85 %) :H_2O = 50 : 0.3 : 49.7 (solvent A) and temperature 26 °C. The detection was carried out at 327 nm.
Data represent the mean values of three independent experiments and standard deviation.

Analysis for resistance profile of LAB against *Geranium* and *Salvia* extracts

The agar well diffusion method was used to detect resistance profile of plant - derived LAB against *G. sanguineum* L. and *S.officinalis* L. extracts. The sterile M17 medium (20ml) was inoculated with test bacteria ($1x10^6$ cfu/ml) and was poured into Petri plates. A sterile cork borer of diameter (8 mm) was used to cut uniform wells in the agar. A 50µl volume of each concentration of the extracts (5 – 150 mg/ml) was added in the wells into M17 plates. The 30% ethanol was used as control. The plant extracts and 30 % ethanol were sterilized by filtration using 0.22 µm syringe filters. All test plates were incubated at 37°C for 48 h. The diameter (mm) of inhibition zones of the extracts was measured. Data represent the mean values of three independent experiments and standard deviation.
The inhibitory activity of *S. officinalis* L. and *G. sanguineum* L. extracts on the growth of the LAB, belonging to the collection of the laboratory and isolated from various dairy products, as well as used in our previous studies (*Streptococcus thermophilus* ST3, *Enterococcus faecium* EF4) was examined.

RESULTS AND DISCUSSIONS

G. sanguineum L. and *Salvia officinalis* L. are plants containing compounds from the group of polyphenols. (Pantev et al., 2006; Martins et al., 2015) Data for content of flavonoids and phenolic acids in *G. sanguineum* L. and *S. officinalis* L. extracts showed significant variety of these biologically active components (Table 1). It was observed a wider range of phenolic compounds in the extracts of *S. officinalis* L., compared to those of *G. sanguineum* L. Furthermore, it was observed also strict specificity of presence of individual phenolic acids in the studied extracts (gallic acid, 3,4-dihydroxy-benzoic acid, 2-hydroxy-benzoic acid – *G. sanguineum* L.; vanillic acid, syringic acid, p-coumaric acid, ferulic acid, rosmarinic acid - *Salvia officinalis* L. A similar trend was established for the content of flavonoids in the studied extracts. Specific flavonoids (myricetin, luteolin, rutin, hyperozide) contained in the extracts of *S. officinalis* L. were not detected in the extracts of *G. sanguineum* L., except for rutin and hyperozide, found in insignificant concentrations (Table 1).

Table 1. Concentration of polyphenolic compounds in the extracts of *G.sanguineum* L. and *S.officinalis* L.

	Compound, mg/g extract	*G.sanguineum* L.		*S.officinalis* L.	
		Hydroethanolic extract	Aqueous extract	Hydroethanolic extract	Aqueous extract
Phenolic acids					
	Gallic acid	2.72 ± 0.07	1.92± 0.05	nd	nd
	3,4-dihydroxy-benzoic acid	0.34 ± 0.01	0.29 ± 0.03	nd	nd
	2-hydroxy-benzoic acid	2.06 ± 0.08	2.50 ± 0.07	nd	nd
	Vanillic acid	nd	nd	0.25 ± 0.01	0.25 ± 0.03
	Syringic acid	nd	nd	0.70 ± 0.05	0.46 ± 0.04
	Caffeic acid	nd	nd	nd	nd
	Chlorogenic acid	nd	nd	nd	nd
	p-Coumaric acid	nd	nd	3.65 ± 0.07	3.01 ± 0.07
	Sinapic acid	nd	nd	nd	nd
	Ferulic acid	nd	nd	0.51 ± 0.05	0.24 ± 0.03
	Cinnamic acid	nd	nd	nd	nd
	Rosmarinic acid	nd	nd	48.69±0.23	22.18±0.17
Flavonoids					
Flavonols	Myricetin	nd	nd	0.13 ± 0.02	0.14 ± 0.01
	Kaempferol	nd	nd	nd	nd
	Quercetine	nd	nd	nd	nd
Flavanone glycoside	Hesperidin	nd	nd	nd	nd
Flavone	Apigenin	nd	nd	nd	nd
	Luteolin	nd	nd	0.04 ± 0.01	0.03 ± 0.01
Quercetin glycoside	Rutin	0.04 ± 0.01	nd	9.96± 0.12	nd
	Hyperozide	nd	0.04 ± 0.01	17.24± 0.13	15.75± 0.14

nd - not detected

The species *S. thermophilus* and *Ent. faecium*, isolated from representatives of the genus *Salvia* (*S. blepharophylla* Brandegee ex Epling, *S. scabiosifolia* Lam., *S. ringens* Sibth. & Sm., *S. officinalis* L.), showed a wide variety concerning their resistance to the extract of *S. officinalis* L. (Table 2). A similar trend was established also in testing the extract of *G. sanguineum* L. on the development of these types of lactobacteria, but isolated from *G. sanguineum* (Table 3). Interspecies as well as interstrain differences in the extent of growth inhibition, depending on the concentration of the tested extracts were found (Table 2, 3).

S. thermophilus strains (Sbf352, Sbf373, Sbf401) isolated from *Salvia,* were inhibited by the ethanol extracts, at low studied concentrations (25-30 mg/ml), while to aqueous extracts the strains demonstrated resistance to all the tested concentrations (20-150 mg/ml) (Table 2).

54 % of *Ent. faecium* strains (isolated from *Salvia*) showed resistance to the ethanol extracts up to a concentration of 100 mg/ml, while insignificant part of the strains (Sof271, Sof277 and Sof279), showed a high sensitivity to the ethanol extracts (40-50 mg/ml). *Ent. faecium* strains showed resistance to the aqueous extracts, throughout the whole studied range. The only exception is *Ent. faecium* Sof279, whose growth was inhibited at the concentration of 40 mg/ml (Table 2).

The controls didn't inhibit the growth of studied strains.

Table 2. Effect of different concentrations of *S. officinalis* L. extracts on the growth of *S. thermophilus* and *Ent. faecium*

Strains	Inhibition Zone (diameter, mm)															
	Hydroetanolic extract, mg/ml								Aqueous extract, mg/ml							
	150	**100**	**75**	**50**	**40**	**30**	**25**	**20**	**150**	**100**	**75**	**50**	**40**	**30**	**25**	**20**
***S. thermophilus*[1]**																
Sbf352	15,0 ±0,5	14,4 ±1,0	12,2 ±0,8	11,0 ±0,6	10,0 ±0,5	9,2 ±0,3	-	-	-	-	-	-	-	-	-	-
Sbf373	19,0 ±1,0	18,0 ±1,1	15,4 ±0,8	13,0 ±0,6	12,4 ±0,6	11,1 ±0,5	9,2 ±0,3	-	-	-	-	-	-	-	-	-
Sbf401	18,2 ±1,0	17,3 ±1,0	15,3 ±1,0	12,2 ±0,6	11,0 ±0,6	9,3 ±0,6	-	-	-	-	-	-	-	-	-	-
***Ent. faecium*[2]**																
Ssf21	10,4 ±0,5	9,2 ±0,3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ssf22	12,3 ±0,5	11,4 ±0,5	10,3 ±0,5	-	-	-	-	-	-	-	-	-	-	-	-	-
Ssf32	10,4 ±0,5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ssf33	9,2 ±0,3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ssf34	10,5 ±0,5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Srs161	9,2 ±0,3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sof271	12,0 ±0,5	11,4 ±0,7	10,3 ±0,5	9,2 ±0,3	-	-	-	-	-	-	-	-	-	-	-	-
Sof277	12,0 ±0,9	11,0 ±0,5	10,0 ±0,4	9,2 ±0,3	-	-	-	-	-	-	-	-	-	-	-	-
Sof279	14,0 ±1,1	13,0 ±0,5	12,4 ±0,6	10,3 ±0,5	9,3 ±0,6	-	-	-	14,5 ±1,0	13,5 ±0,8	12,0 ±0,5	10,0 ±0,5	9,3 ±0,6	-	-	-
Sol284	9,3 ±0,6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sos301	9,5 ±0,5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sos312	11,5 ±1,2	10,3 ±0,5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sos328	10,5 ±0,5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1] *S. thermophilus* strains isolated from *Salvia* species (*S. blepharophylla* Brandegee ex Epling); [2] *Ent. faecium* strains isolated from Salvia species (*S. scabiosifolia* Lam., *S. ringens* Sibth. & Sm., *S. officinalis* L.) (-) – resistant, without zone of inhibition;

Table 3. Effect of different concentrations of *G.sanguineum* L. extracts on the growth of *S. thermophilus* and *Ent. faecium*

Strains	Inhibition Zone (diameter, mm)																	
	Hydroetanolic extract, mg/ml									Aqueous extract, mg/ml								
	75	50	30	25	20	15	10	8	5	75	50	30	25	20	15	10	8	5
***S. thermophilus*[1]**																		
Gsf60	15,5 ±0,5	13,0 ±0,8	10,8 ±0,5	10,0 ±0,5	9,2 ±0,3	-	-	-	-	15,0 ±1,2	11,2 ±0,6	10,5 ±0,5	10,1 ±0,7	9,2 ±0,3	-	-	-	-
Gsf65	14,3 ±0,6	12,0 ±0,9	10,2 ±0,6	9,3 ±0,6	9,2 ±0,3	-	-	-	-	14,1 ±0,7	13,5 ±0,5	10,0 ±0,5	9,3 ±0,6	9,2 ±0,3	-	-	-	-
Gsf69	14,1 ±0,7	13,5 ±1,0	12,2 ±0,7	11,0 ±0,6	10,5 ±0,5	9,2 ±0,3	-	-	-	13,1 ±0,5	12,4 ±1,2	11,0 ±0,5	-	-	-	-	-	-
Gsf76	13,1 ±0,5	11,0 ±0,5	9,3 ±0,6	9,2 ±0,3	-	-	-	-	-	11,2 ±0,6	9,3 ±0,6	9,2 ±0,3	-	-	-	-	-	-
***Ent. faecium*[2]**																		
Gsf123	12,0 ±1,0	10,5 ±0,5	9,3 ±0,6	-	-	-	-	-	-	12,0 ±1,0	11,0 ±0,5	9,3 ±0,6	-	-	-	-	-	-
Gsf124	12,0 ±1,0	10,2 ±0,5	9,3 ±0,6	-	-	-	-	-	-	11,0 ±0,5	10,0 ±0,3	9,2 ±0,3	-	-	-	-	-	-
Gsl123	12,4 ±0,9	11,2 ±0,5	10,4 ±0,4	10,0 ±0,5	-	-	-	-	-	10,4 ±0,5	9,2 ±0,3	-	-	-	-	-	-	-
Gsl124	13,2 ±1,2	11,2 ±0,5	10,3 ±0,5	9,3 ±0,6	-	-	-	-	-	12,5 ±1,0	11,3 ±0,9	-	-	-	-	-	-	-
Gsl213	16,3 ±0,9	14,3 ±1,1	13,0 ±0,5	12,5 ±1,0	11,2 ±0,5	10,4 ±0,4	10,0 ±0,5	9,2 ±0,3	-	15,0 ±0,5	13,5 ±1,0	13,1 ±0,5	11,2 ±0,6	10,3 ±0,5	9,5 ±0,5	9,2 ±0,3	-	-
Gsf313	15,3 ±1,2	13,5 ±1,0	11,5 ±1,2	11,0 ±0,4	11,0 ±0,4	10,5 ±0,5	10,0 ±0,5	-	-	15,0 ±0,5	13,0 ±0,5	12,0 ±1,0	11,4 ±0,8	10,0 ±0,5	9,3 ±0,6	9,2 ±0,3	-	-
Gsl312	16,1 ±0,9	14,5 ±0,5	12,0 ±1,0	11,0 ±0,5	10,5 ±0,2	10,3 ±0,5	9,2 ±0,3	-	-	15,2 ±0,6	14,3 ±0,7	12,0 ±1,0	11,0 ±0,5	10,0 ±0,3	9,2 ±0,3	9,2 ±0,3	-	-
Gsf2115	12,5 ±1,0	11,0 ±0,4	10,4 ±0,4	10,0 ±0,5	9,3 ±0,6	-	-	-	-	12,1 ±0,5	11,2 ±0,6	10,0 ±0,5	9,5 ±0,5	9,2 ±0,3	-	-	-	-
Gsf2101	12,0 ±0,5	11,2 ±0,5	10,2 ±0,5	9,3 ±0,6	9,2 ±0,3	-	-	-	-	12,5 ±1,0	11,1 ±0,6	10,3 ±0,5	10,0 ±0,5	9,3 ±0,6	-	-	-	-
Gsl2227	11,0 ±0,5	10,5 ±0,5	9,3 ±0,6	9,2 ±0,3	-	-	-	-	-	11,4 ±0,8	10,0 ±0,3	9,3 ±0,6	9,2 ±0,3	-	-	-	-	-
Gsl2212	12,0 ±1,0	11,5 ±0,5	10,0 ±0,5	9,2 ±0,3	-	-	-	-	-	11,5 ±0,5	10,5 ±0,5	10,4 ±0,4	9,2 ±0,3	-	-	-	-	-

[1] *S. thermophilus* strains isolated from *G. sanguineum* L.; [2] *Ent. faecium* strains isolated from *G. sanguineum* L.; (-) – resistant, without zone of inhibition;

The ethanol and the aqueous extracts of bloody geranium showed a similar inhibitory influence on *S. thermophilus* and *Ent. faecium* strains, isolated from *Geranium*. Inhibition of *S. thermophilus* strains was observed at a concentration of ethanolic extract between 15-25 mg/ml and of aqueous extract between 20-30 mg/ml (Table 3). *Ent. faecium* strains showed resistance to lower concentrations – 5 - 25 mg/ml and 8- 30 mg/ml, of ethanol and aqueous extract, respectively (Table 3). The controls didn't inhibit growth of studied strains. The available information concerning studies of resistance profile of plant - derived LAB to relevant plant extracts is scarce (Saguibo et al, 2012).

Most collectives study the effect of various plants, spice, vegetable extracts on LAB species, but not from plant origin (Sagdic et al., 2003; Sagdic et al, 2005; Michael et al., 2010; Saguibo and Elegado, 2012; Ekren et al., 2013). It was also examined the influence of different concentrations of the extracts on the growth of LAB isolated from dairy products (*Streptococcus thermophilus* ST3, *Enterococcus faecium* EF4). It was observed that LAB of diary origin demonstrate similar resistance to the tested plant extracts regarding plant - derived LAB.

CONCLUSIONS

Most of the LAB strains (*S. thermophilus*, *Ent. faecium*) isolated from *Geranium* showed a similar resistance profile to those of dairy origin, both to the ethanol and to aqueous extracts of *Geranium*. Exceptions are 2 strains of *Ent. faecium* (Gsl123, Gsl124), which demonstrated a higher level of resistance to the aqueous extract.

S. thermophilus and *Ent. faecium*, isolated both from *Salvia* and from dairy products, showed resistance to the aqueous plant extract. Regarding the ethanol extract, *S. thermophilus* of plant and dairy origin also showed similar behavior - high level of inhibition.

The trend for total similarity of resistant profiles is not valid for *Ent. faecium*, isolated from *Salvia* species and dairy products - about 60% of *Ent. faecium*, showed higher level of resistance compared to *Ent. faecium* from diary origin.

Probably some strains of plant – derived LAB are more resistant to extracts of the plant, from which they are isolated, in comparison to those isolated from dairy products. Out of all isolated plant-derived LAB, highest resistance was reported for the strains of *Ent. faecium* isolated from *Salvia*.

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