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BIODIVERSITY AND BIOTECHNOLOGICAL POTENTIAL OF LACTIC ACID BACTERIA

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Abstract

Lactic acid bacteria (LAB) constitute a heterogeneous group of industrially important bacteria that are used to produce fermented foods and beverages, using various substrates, such as milk, vegetables, cereals, meat, cocoa beans etc.The most important advantage of LAB, making them suitable for the use in food biotechnology, is that they are generally recognized as safe (GRAS). LAB have been shown to contribute to the improvement of the shelf life of fermented foods, due to the production of a wide variety of compounds, acting in a synergistic way to prevent or eliminate microbial contamination. In fermented foods, LAB also contribute to the nutritional and organoleptic characteristics of the final products and they are traditionally used as starter cultures for the industrial production of many types of foods and beverages. The so-called "functional foods" concept was recently proposed and has shown a remarkable growth over the last few years. Such foods should promote well-being and health, while at the same time should reduce the risk of some major chronic and degenerative diseases, such as cancer, cardiovascular diseases, obesity and gastrointestinal tract disorders. Therefore, an increasing demand exists for new functional starter strains that show desirable effects on the product characteristics. They should possess at least one inherent functional property, contributing to food safety and/or offering one or more organoleptic, technological, nutritional, or health advantages. Traditionally fermented foods are the best places to look for such microorganisms with potential applications in food industry and health. In Romania, fermented foods are still produced, at a large extent, in a traditional way, depending on local and regional traditions and on the indigenous microbiota present or selected by the environment or technology used (no starter cultures are added). Some of these products, mainly fermented vegetables and cereals are known for their positive effect on human organisms, especially due to the high vitamin and mineral content. The present paper will present our most important findings on the diversity of LAB in traditional Romanian fermented foods, but also on the isolation and characterization of some metabolites produced by these bacteria, with potential applications in food biotechnology.

***Keywords**: lactic acid bacteria, bacteriocins, exopolysaccharides, functional foods.*

INTRODUCTION

The group of lactic acid bacteria (LAB) gained lately a lot of interest, based on their use since ancient time in the fermentation of many substrates, such as milk, meat, fish, vegetables, cereals, cocoa beans etc. (Doyle and Beuchat, 2007; Wood and Holzapfel, 1995; Wood, 1997), as the first natural preservation method of the raw materials. The most important advantage of LAB, making them suitable for the use in food biotechnology, is that they are generally recognized as safe (GRAS); they are harmless for humans, on the contrary, having many beneficial health effects.

LAB have been shown to contribute to the improvement of the shelf life of fermented foods, due to the production of a wide variety

of compounds (organic acids, ethanol, hydrogen peroxide, bacteriocins, antibiotic-like peptides etc.), acting in a synergistic way to prevent or eliminate microbial contamination (De Vuyst & Vandamme, 1994). Based on this antimicrobial effect, LAB have been proposed to be used as bioprotective agents against foodborne pathogens (Leverentz et al., 2006; Trias et al., 2008).
In fermented foods, LAB also contribute to the nutritional and organoleptic characteristics of the final products and they are traditionally used as starter cultures for the industrial production of many types of foods and beverages. These properties and the metabolites involved were extensively studied during the last decades. For instance, vitamins, low-calorie sugars, and bioactive peptides produced by LAB are typical examples of nutraceuticals - food components with a claimed medical or health benefit (El Sohaimy, 2012). Moreover, certain LAB strains, the so-called probiotics, display beneficial health properties, including, amongst many other, equilibration of the intestinal microbiota, antimicrobial activity, improving the digestibility of ingested food, anti-tumor properties and ability to neutralize toxic compounds, immunomodulating and cholesterol-lowering effects (Wood, 1999). On the other hand, many LAB strains have the ability to produce exopolysaccharides (EPS), with an important role in the rheology and texture properties of fermented food products, and thus of interest for food applications as *in situ* produced, natural bio-thickeners (De Vuyst&Vaningelgem, 2003; Mozzi et al., 2010). Some EPS and oligosaccharides produced by LAB might also have prebiotic activity (Ruijssenaars, 2000; Salazar, 2008), contributing to the promotion of human gastrointestinal health.
The so-called "functional foods" concept was recently proposed and has shown a remarkable growth over the last few years (Annunziata &Vecchio, 2011). Such foods should promote well-being and health, while at the same time should reduce the risk of some major chronic and degenerative diseases, such as cancer, cardiovascular diseases, obesity and gastrointestinal tract disorders (Mozzi et al., 2010). Therefore, an increasing demand exists for new functional starter strains that show desirable effects on the product characteristics. They should possess at least one inherent functional property, contributing to food safety and/or offering one or more organoleptic, technological, nutritional, or health advantages (Leroy&De Vuyst, 2004).
Traditionally fermented foods are the best places to look for such microorganisms with potential applications in food industry and health. In Romania, such foods are still consumed at a large extent and some of them, mainly fermented vegetables and *borș* (fermented wheat bran) are known for their positive effect on human organisms, especially due to the high vitamin and mineral content.
This short review will present our most important findings on the diversity of LAB in traditional Romanian fermented foods, including dairy products and fermented vegetables, but also on the isolation and characterization of some metabolites produced by these bacteria, with potential applications in food biotechnology.

BIODIVERSITY OF LAB IN ROMANIAN FERMENTED FOODS

In Romania, many fermented foods (dairy, vegetables, even cereals – *borș*) are still producedin a traditional way, depending on local and regional traditions and on the indigenous microbiota present or selected by the environment or technology used (no starter cultures are added). During several years, in the framework of several projects, the LAB diversity of such traditional fermented foods, collected from farmhouses, local markets, and monasteries, was investigated. The bacterial isolates were identified to species level using both clasical methods (Gram-staining, cell morphology, catalaseproduction etc.) and modern techniques (SDS-PAGE of total cell proteins, $(GTG)_5$-PCR fingerprinting, 16S rRNA sequencing etc.).
The biodiversity of Romanian raw milk and traditional fermented dairy products proved to be characterized by lactococci, with *Lactococcus lactis* as the most frequent species, leuconostocs, and enterococci (Zamfir et al., 2006) (Figure 1). Among the latter, the new species *E. saccharominimus* was found (Vancanneyt et al., 2004). Lactobacilli are

found, but in low numbers. During the study, it was proven that *Leuconostoc argentinum* is a later synonym of *Leuconostuc lactis* (Vancanneyt et al., 2006). The variability of the LAB strains isolated from the samples tested reflected their artisan production. Based on our findings, Romanian fermented milks could be assigned to the group of mesophilic fermented milks, according to the classification of Robinson & Tamime (1990), with a dominating microbiota consisting of lactococci and leuconostocs. This type of fermented milks is typical for Scandinavian and Central and Eastern European countries (Oberman& Libudzisz, 1998).

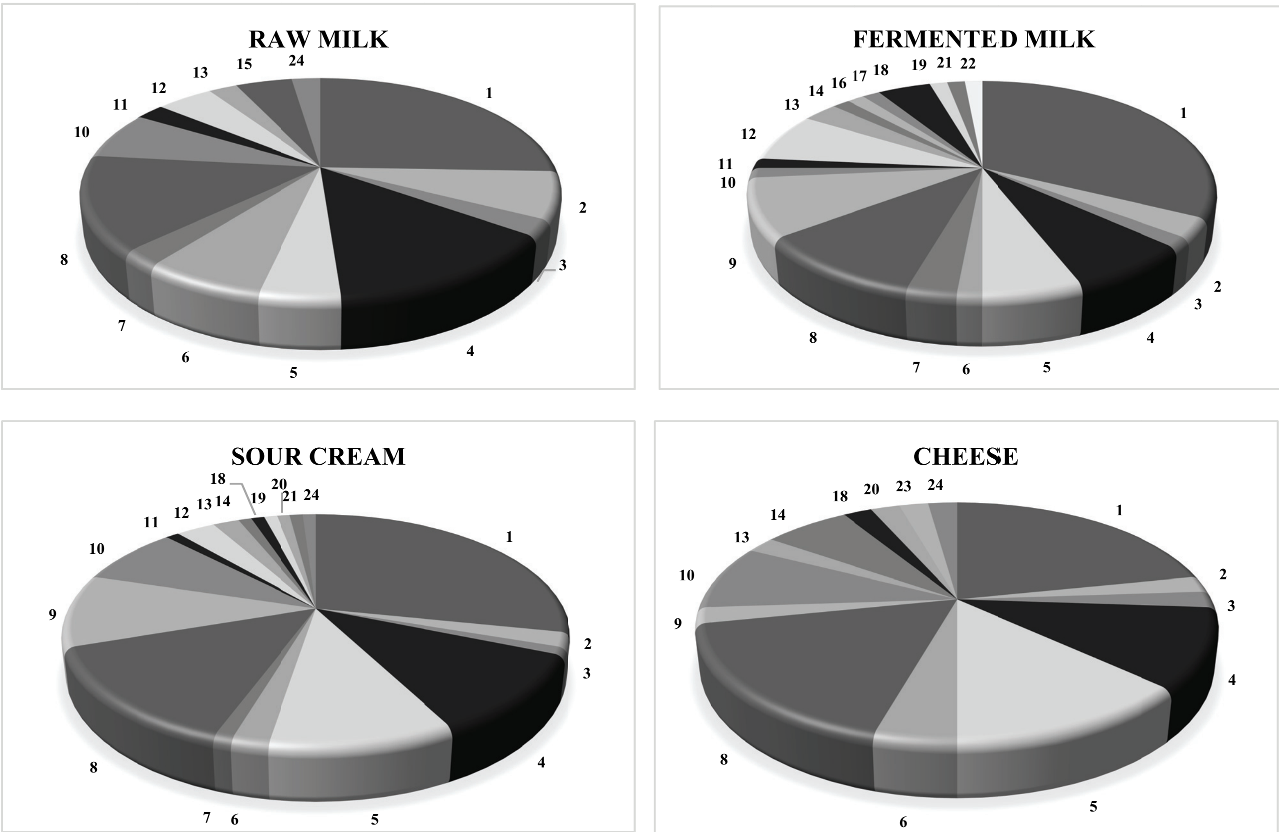

Figure 1. Incidence of LAB taxa in raw milk and fermented dairy products investigated. Numbers represents: 1 - *Lactococcus lactis* subsp. *lactis*; 2 -*Lactococcus lactis* subsp. *cremoris*; 3 - *Lactococcus garviae*; 4 - *Enterococcus durans*; 5 - *Enterococcus faecium*; 6 - *Enterococcus faecalis*; 7 - *Enterococcus sacchar ominimus*; 8 - *Leuconostoc mesenteroides*; 9 - *Leuconostoc pseudomesenteroides*; 10 - *Leuconostoc lactis*; 11 - *Leuconostoc citreum*; 12 - *Lactobacillus plantarum*; 13 - *Lactobacillus paracasei* subsp. *paracasei*; 14 - *Lactobacillus curvatus*; 15 - *Lactobacillus animalis*; 16 - *Lactobacillus helveticus*; 17 - *Lactobacillus kefir*; 18 - *Lactobacillus fermentum*; 19 - *Lactobacillus delbrueckii* subsp. *bulgaricus*; 20 - *Lactobacillus brevis*; 21 - *Streptococcus thermophilus*; 22 - *Streptococcus bovis*; 23 - *Weissella viridescens*; 24 - unidentified strains

Concerning the fermented vegetables, culture-dependent and culture-independent analyses of end-samples of various spontaneous fermentation scarried out in farmhouses in central Romania revealed *Lactobacillus plantarum* and *Lactobacillus brevis* as the most frequently isolated LAB species (Table 1). *Leuconostoc mesenteroides* and *Leuconostoc citreum* were also found in high numbers (Wouters et al., 2013). In parallel, two fermentations (cauliflower solely and a mixture of cauliflower, carrots and green tomatoes) were followed asa function of time to determine their community dynamics andmetabolite kinetics. These fermentations were characterised by the omnipresence of *Lactobacillus plantarum*. Based on the LAB diversity, the two fermentations seemed to follow a three steps pattern: a first phase with a wide LAB species diversity (including *Weissella kimchi, Leuconostoc citreum, Lactobacillus brevis, Lactobacillus sakei/curvatus*) followed by a phase with more adaptedspecies, in turn followed by a phase with the most prevailing ones (*Lactobacillus brevis* and *Lactobacillus plantarum*). Metabolite target analysis revealed that glucose andfructose were mostly depleted at the end of fermentation. The main products of carbohydrate metabolism were lactic acid,acetic acid, ethanol and small amounts ofmannitol, indicating heterolactate fermentation. Since *Lactobacillus brevis* and *Lactobacillus plantarum* are the most prevalent in vegetable fermentations, at least in the end of the process, strains belonging to these species might be further used as starter culture for controlled fermentations (Wouters et al., 2013).

Table 1. LAB species identified in traditional fermented vegetables

Fermentation	Time of sampling	pH (brine)	LAB species
Cabbage	1 month	3.59	*Lb. plantarum, Lb. brevis,* unidentified
Cabbage	2 months	3.37	*Lb. plantarum, Lb. brevis*
Cabbage and beet	1 month	3.67	*Lb. plantarum, Lb. brevis*
Cucumbers	1 month	3.56	*Lb. plantarum, Lb. brevis*
Cucumbers	2 months	3.42	*Lb. plantarum/pentosus*
Cucumbers	3 months	3.50	-
Cucumbers and cauliflower	1.5 months	3.57	*Lb. plantarum, Lb. brevis*, unidentified
Cucumbers and plums	2 months	3.68	*Lb. plantarum, Lb. brevis*, unidentified
Green tomatoes	1 month	3.93	*Lb. plantarum, Lb. brevis, Leuc. citreum*
Green tomatoes and carrots	1 month	3.77	*Lb. plantarum, Leuc. citreum*
Green tomatoes and cucumbers	2 months	3.71	*Lb. plantarum, Lb. brevis, Lb. plantarum/pentosus*
Green tomatoes, cauliflower, and carrots	2 months	3.56	*Lb. plantarum, Lb. brevis, Lb. plantarum/pentosus*
Green tomatoes, celery and carrots	2 months	3.82	*Lb. plantarum, Lb. brevis, Lb. plantarum/pentosus*
Green tomatoes apples, pears, cucumbers, and beet	0.5 months	4.18	*Lb. plantarum, Leuc. mesenteroides,* unidentified
Pepper filled with chopped cabbage and carrots	2 months	3.44	*Lb. plantarum, Lb. brevis,* unidentified

BACTERIOCINS FROM LAB

LAB are able to synthetise several compounds with antibacterial activity. Amongst them, bacteriocins received a lot of interest and are well characterized. They are ribosomally synthetized antimicrobial peptides or proteins that inhibit growth of other bacteria, usually closely related to the producing organism (De Vuyst&Leroy, 2007). The antibacterial spectrum includes, however, several spoilage microoorganisms and food-borne pathogens such as *Bacillus cereus, Clostridium botulinum, Clostridium perfringens, Listeria monocytogenes,* and *Staphylococcus aureus* (De Vuyst and Leroy, 2007; Sip et al., 2012; Zendo, 2013). Furthermore, many bacteriocins are heat stable, making them applicable in combination with heat treatment. Finally, they are food stable, biodegradable, digestible, safe to health and active at low concentrations (De Vuyst&Vandamme, 1994). Because of all theseadvantages, bacteriocins produced by lactic acid bacteria might find applications in food industry, as additives used to prolong the shelf life of many foods.

Several bacteriocin-producing LAB strains have been selected during our studies. Acidophilin 801, produced by *Lactobacillus acidophilus* IBB801 (Figure 2), isolated from dairy products, has been extensively characterized. The inhibitory activity of *L.*

acidophilus IBB 801 is restricted to closely related lactobacilli, but under certain conditions, it also inhibits the growth of *E. coli* Row, *S. panama* 1467, and *Klebsiella pneumoniae* K33 (Zamfir et al., 1999). While catalase does not affect the activity, incubation of bacteriocin samples with trypsin, pronase, or proteinase K at 37°C for 1 h completely destroyes the antibacterial activity. Moreover, acidophilin 801 is strongly heat resistant, a small inhibitory activity being observed even after incubation for 30 min at 121°C, and rezistant to pH variations. The bacteriocin was purified by reversed-phase highperformance liquidchromatography and the molecular mass was estimated to about 6500 Daby sodiumdodecylsulfate-polyacrylamide gel electrophoresis (Zamfir et al., 1999).

Whereas most antibiotics (usually classified as secondary metabolites) are synthesised during the stationary growth phase, almost all bacteriocins produced by lactic acid bacteria display primary metabolite kinetics (Lejeune et al., 1998), including acidophilin 801 produced by *L. acidophilus* IBB 801 (Zamfir et al., 2000). The bacteriocin is produced during the exponential growth phase with a maximum in the middle or near the end of this phase. Moreover, when different growth conditions were compared as to their capacity to support bacteriocin production by *L. acidophilus* IBB 801, it turned out that growth and bacteriocin production were enhanced when the medium was supplemented with 1% (m/v) of yeast extract, or in the presence of some amino acids (glutamic acid or leucine). Growth and bacteriocin production were also enhanced when fermentations at constant pH (6.0) were carried out. Maintaining the pH at a value optimal for growth increases the specific growth rate, prolongs the exponential growth phase provided enough of the energy source is present, and results in higher biomass amounts and, because of its growth-associated production kinetics, higher bacteriocin titres. On the other hand, mild stress conditions (low concentration of NaCl) orgrowth at 10°C, resulted in an increase of the specific bacteriocin production (Zamfir & Grosu-Tudor, 2009).

Concerning the mode of action, acidophilin has a bactericidal, concentration-dependent effect towards sensitive strains, without causing concomitant cell lysis of the indicator cells.

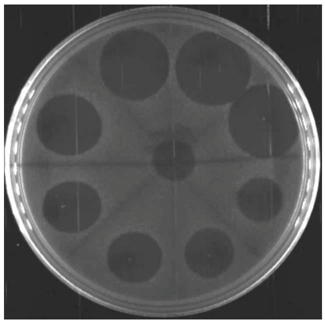

Figure 2. Antibacterial activity of *Lactobacillus acidophilus* IBB801 supernatants against *Lactobacillus delbrueckii* subsp. *bulgaricus* (serial dilutions from 1 to 1/256)

The most important step in its mode of action seems to be the pore formation that causes the release of intracellular molecules. Consequently, the glycolytic rate is reduced and hence many vital processes might be blocked. All these effects cause growth inhibition and result in the ultimate death of sensitive cells (Zamfir et al., 2007).

EPS PRODUCTION BY LAB

In fermented foods, LABare not only important because they acidify and hence preserve food products from spoilage, but also because of their contribution to the organoleptic properties of the fermented foods. The contribution of LAB to the texture development of yogurts and other fermented milks, low-fat cheeses, and dairy desserts is well known. This is partly due to the production of exopolysaccharides (EPS), which are long-chain polysaccharides, composed of one type (homopolysaccharides-HoPS) or more different monosaccharides (heteropolysaccha-rides-HePS), released by the bacteria in their surroundings (De Vuyst & Vaningelgem, 2003). EPS increase the viscosity and firmness, improve the texture, reduce susceptibility to syneresis, and contribute to the mouthfeel of low-fat products (De Vuyst&Vaningelgem, 2003; Mozzi et al., 2010). *In situ* production of EPS by LAB to get a desired texture and mouthfeel of some dairy products is being explored, in order to replace polysaccharides from plants (starch, pectin, guar gum, alginate) or animals (gelatine), currently in use. This would lower the production costs and would lead to a more natural product.In this context, a rational screening for novel EPS from particular LAB strains that are characterized by a unique structure or molecular mass is of utmost importance regarding their applications.

Several LAB strains, isolated from traditional Romanian dairy products and fermented vegetables, have been shown to produce both HoPS and HePS, in various amount (Table 2). In general, HePS are produced in much lower amounts compared with HoPS, and also compared with other bacterial polymer producers, such as the aerobic *Xanthomonas campestris* producing xanthan (Ruas-Madiedo et al., 2009).

Table 2. EPS production by LAB isolated from fermented foods

Producing strain	Source of isolation	EPS yield (g/l)	Molecular mass (KDa)	Monomer composition
S. thermophilus ST111	Yogurt	0.05[a]	> 2000	2.5Gal:1.0Rha
S. thermophilus ST110	Yogurt	0.012[a]	> 2000	3.9Gal:1.2Glc:1.0GalN(Ac)
S. thermophilus ST113	Yogurt	0.023[a]	210	1.7Gal:3.9Glc:1.0GalN(Ac)
S. thermophilus ST114	Yogurt	0.014[a]	> 2000	3.7Gal:2.4Glc:1.0Rha
S. thermophilus S509	Yogurt	0.036[a]	> 2000	3.0Gal:1.0Rha
S. thermophilus SR1	Yogurt	0.028[a]	18	1.6Gal:1.0Glc:1.0Rha
L. lactis 1.8	Raw milk	4.62[b]	> 5000	Glc
Leuc. citreum 1.10	Raw milk	15.48[b]	> 5000	Glc
Leuc. citreum 1.11	Raw milk	13.75[b]	> 5000	Glc
Leuc. citreum 1.12	Raw milk	15.40[b]	> 5000	Glc
Leuc. citreum 2.8	Fermented milk	16.30[b]	> 5000	Glc
Leuc. citreum 4.11	Raw milk	16.71[b]	> 5000	Glc
Leuc. pseudomesenteroides 20.6	Raw milk	2.24[b]	> 5000	Glc
Leuc. mesenteroides 21.2	Raw milk	13.89[b]	> 5000	Glc
Weissella confusa/cibaria 38.2	Sour cream f.v.	2.74[b]	> 5000	Glc
Leuc. citreum 177	f.v.	19.43[b]	> 2000	Glc
Leuc. citreum 52	f.v.	21.13[b]	> 2000	Glc
Unidentified strain 37	f.v.	4.68[b]	> 2000	Glc
Leuc. mesenteroides 2	f.v.	2.64[b]	> 2000	Glc
Leuc. mesenteroides 368	f.v.	0.63[b]	> 2000	Glc
Weisella viridescens 28	f.v.	0.03[b]	> 2000	Glc
Lb. curvatus/sakei 29		0.02[b]	> 2000	Glc

[a]EPS yield in milk medium
[b]EPS yield in filtered MRS medium with 50g/l sucrose
f.v. = fermented vegetables
Gal=galactose, Glc=glucose, Rha=rhamnose, GalN (Ac)=(N-acetyl)-galactoseamine

With only two exceptions, the EPS isolated from the selected strains are large polymers, with a molecular mass over 2000 or even 5000 KDa. Moreover, most of the EPS are HoPS, composed of glucose solely, and only six are HePS, composed of two or three different monosaccharides (Table 2). For the HoPS produced by LAB isolated from dairy products, the linkage analysis revealed the dominance of α-1.6 linkage, amongst other different types of α-linkages (Grosu-Tudor et al., 2013a). The structure of the repeating unit of the HePS produced by *S. thermophilus* ST111 has been also elucidated by NMR spectroscopy (De Vust et al., 2003).

EPS production was, in general, strictly correlated with growth, the optimal growth conditions resulting in the highest EPS yields. Higher yields were also obtained when the producing strains were grown at controlled pH, or in milk supplemented with lactalbumin hydrolysate (Vaningelgem et al., 2004b; Zamfir & Grosu-Tudor, 2014). Finally, six selected EPS (both HoPS and HePS) were tested for their potential prebiotic effect, in *in vitro* studies simulating the passage through the upper gastrointestinal tract. The results were promising, since none of the tested EPS was degraded by the low pH or treatment with pepsin and pancreatin. On the other hand, in low scale fermentations, it was shown that some of these EPS could be metabolized by some beneficial bacteria, such as *Bifidobacteria* (Grosu-Tudor et al., 2013b). Our work provides evidence that natural environments (such as traditional fermented foods) are rich in biodiversity and can be important sources for new strains and species within the LAB group. Among these LAB one can find strains with functional properties (such asbacteriocin or EPS production, pro- or prebiotic effect) and hence with potential biotechnological applications.

β-D-Gal*p*-(1-6)-β–D-Gal*p*
1
↓
4
2)-α-L-Rha*p*-(1→2)-α-D-Gal*p*-(1→ 3)-α-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→3)-α-L-Rha*p*-(1→

Figure 3. Structure of the repeating unit of the HePS produced by *S. thermophilus* ST111

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