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Original paper

Screening of lactic acid bacteria from spontaneously fermented products of Romania

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Abstract

Lactic acid bacteria (LAB) are an important part of the microbiota of fermented food and beverages from all over the world, their contribution to preservation and safety of food to which they also confer specific attractive textures and aromas being essential. Certain selected strains were characterized in terms of their probiotic activity, positively reflecting on consumers' health. LAB can also be regarded as sources of functional compounds such as vitamins, enzymes a. o., with applications in the food and in the pharmaceutical industry. The screening of LAB from local or traditional fermented foods for their biotechnological potential continues to be a very promising direction of research. The aim of the present study was the characterization of newly isolated LAB strains from various spontaneously fermented foods and beverages locally produced in Romania and the evaluation of their biotechnological characteristics. A total of 130 LAB strains were examined for their acidification potential, for the production of organic acids, exopolysaccharides (EPS) and diacetyl, for their citrate fermentation capacity, and for their proteolytic and lipolytic activity. A series of strains that were considered to hold potential for further applications could be selected in view of further, more detailed studies.

Keywords Lactic acid bacteria screening, local fermented foods and beverages

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Introduction

The contemporary landscape of food trade tends to homogenization of products and even of food habits (PINGALI, [1]). Nevertheless, the contrary tendency of promoting the diversity of local (i. e. locally produced) and traditional (i. e. specific for a certain local culture) food does also exist. Migration, as well as food tourism (the latter being an important part of nowadays tourism market, SU and HORNG, [2]), provide unmediated dissemination of local and traditional foods. Fermented foods and beverages – today considered groupable in nine classes, fermented cereals, vegetables and bamboo shoots, legumes, roots/tubers, milk products, fermented and preserved meat products, fermented, dried and smoked fish products, and alcoholic beverages (TAMANG & al., [3]) – are very diverse and spread all over the world. Fermentation may involve yeasts, acetic acid bacteria, lactic acid bacteria (LAB), propionic acid bacteria, members of the genus *Bacillus*, and molds; associations among diverse strains might occur, their stability ensuring the properties of each product, with specific aromas and textures (MARCO & al., [4]).

Most of the lactic acid bacteria of biotechnological interest are part of the families *Enterococcaceae, Leuconostocaceae, Lactobacillaceae,* and *Streptococcaceae*, the more prominent genera being *Enterococcus (E.), Oenococcus (O.), Leuconostoc (Leuc.), Lactobacillus (Lb.), Pediococcus (P.), Lactococcus (Lc.), Streptococcus (S.)*, with several hundred strains described till nowadays. The members of the above enumerated taxa are forming an ecologically diverse group of Gram-positive facultative anaerobic, homofermentative non-sporing rods and cocci able to yield lactic acid as the main product of the carbohydrate metabolism; *sensu lato*, the LAB group also comprises the genus *Bifidobacterium* (class *Actinobacteria*) whose members are heterofermentative producers of lactic acid, some of them employed as probiotics (KHANDELWAL & al., [5]).

Probiotic LAB strains are beneficial for human health. Improving of lactose digestion, protection against diarrhea of various aetiologies and local and general enhancement of the immune function number among the outcomes correlating with probiotics intake, whilst other claims such as prevention of intestinal and urogenital infections, lowering of serum cholesterol levels, anti-diabetic, anti-obesity, anti-inflammatory and angiogenic effects or cancer suppression by means of probiotics consumption need further examination (see MICHAIL & al., [6]; WEDAJO, [7]; SO & al., [8]; KERRY & al., [9]).

The probiotics beneficial effect to health has recently led to a significant increase in research interest in their use to modulate the gut microbiota (VAMANU & al. [10]). Numerous studies have reported beneficial interactions between commensal microbiota and the human body; an imbalance of the human gut microbiota composition can lead to degenerative diseases, diabetes and obesity or cardiovascular disease so the potential use of selected probiotic bacteria species and their strains is desperately needed for the prevention and treatment of such human and animal diseases (VAMANU & al. [11], AZAD & al. [12]).

Lactic acid fermentation is one of the traditional means of ensuring not only preservation of food, but also its safety, warranted by the antagonistic effect of various compounds resulted from the activity of LAB (organic acids, carbon dioxide, hydrogen peroxide, ethanol, diacetyl and bacteriocins), which also leads to the lowering of the redox potential and to nutrient depletion, circumstances unfavorable for the development of both spoilage microbiota and pathogens (DROSINOS and PARAMITHIOTIS, [13]). Lactic acid bacteria can also be regarded as a valuable source of functional ingredients, such as vitamins, enzymes (amylases and peptidases, that might be used in controlled processes for improving the sensorial quality of food by impacting on the texture and flavour), polysaccharides (with applications in the food and pharmaceutical industry and not only), and compounds with the potential to be used as low-calorie sweeteners (FLOROU-PANERI & al., [14]).

Fermented foods from everywhere represent a huge pool of microorganisms harboring an immense potential for controlled biotechnological applications and studying of the microbiota of local and traditional food, that has commenced decades ago, continues to be a matter of special interest (ARSIC & al. [15], ADIGUZEL & al. [16], TUNCER & al [17], BRAJDES &al. [18], GROSU-TUDOR & al [19]). An efficient protocol for studying a traditional fermented product should comprise product detailed investigation including isolation and identification of cultivable organisms, along with assessment of their role in the production process, eventually leading to the optimization of the original process by the use of a starter incorporating the strain or the strains found to be relevant (TAMANG & al., [20]). Optimization by using starters is also thought to increase product safety (CAPOZZI & al., [21]). Probiotic development and use require as well a precise knowledge of the strains and the certainty that the commercial product keeps its identity, which is a must especially when the probiotic is administered to children or likely to be recommended as part of the dietary management in serious health conditions (DE SIMONE, [22]).

Several LAB taxa were identified in naturally fermented foods of Romania – *Lc. lactis*, *Lb. plantarum*, *E. durans*, isolated from dairy products together with the novel species *E. saccharominimus* (VANCANNEYT & al., [23]), *Lb. amylolyticus*, *Lb. oris* (from fermented cereals) (GROSU-TUDOR & al., [24]), *Leuc. citreum, Lb. brevis*, *Leuc. mesenteroides,* and again *Lb. plantarum* (isolated from fermented vegetables) (PELINESCU & al., [25], GROSU-TUDOR & al. [26]), some strains showing a promising potential as probiotics.

The aim of this study was the characterization of newly isolated LAB strains from various spontaneously fermented foods and beverages of Romania by evaluation of their biotechnologically relevant traits.

Materials and Methods

Microbial strains. 130 LAB strains were isolated from fermented cereals (rye, wheat, 35 strains), vegetables and roots (cucumbers, tomatoes, cabbage, carrots, beetroot, 25 strains; European elder juice, apple juice, orange juice, grape must 20 strains), milk products (yogurt, kefir, cheese, sour cream, 50 strains). Strains were purified by streaking on MRS agar (DE MAN & al., [27]) and stored at -70ºC in MRS broth supplemented with 20% (v/v) glycerol. For further testing, each bacterial strain was sub-cultured (1% v/v) in MRS broth for 24 h at 37ºC

Acidification potential and production of organic acids. In order to evaluate the acidifying capacity, overnight cultures were centrifuged at 10.000 rpm for 3 minutes, washed with distilled water, inoculated (1% v/v) in 10 mL UHT milk 0.1% fat and incubated at 37ºC. Measurements of pH values were carried out at 6h, 24h and 48h after inoculation using pH indicator strips. Also, overnight LAB cultures were spotted (10µL) on MRS-$CaCO_3$ agar plates and incubated for 24-48h at 37ºC. Production of organic acids was indicated by a halo around colonies and the diameter of the clear zone (mm) was measured at the end of the incubation time (DE VUYST and VANDAMME, [28]).

Exopolysaccharide assay. Overnight LAB cultures were spotted (10µL) on modified MRS agar plates with 2% sucrose added, and incubated at 37ºC. The EPS producers were selected by their mucoid or ropy appearance and subjected to the pick test (RICCIARDI & al., [29]) at 24h, 48h and 72h after inoculation.

Citrate-fermenting capacity. The citrate test was performed on KMK medium (KEMPLER and MC KAY, [30], see also DRICI & al., [31]) containing nonfat milk (10 mL UHT milk 0.1% fat), glucose (5 g L^{-1}), casein hydrolysate peptone (2.5 g L^{-1}) and agar (15 g L^{-1}). pH was adjusted to 6.6 and the medium was sterilized for 12 min at 115ºC and kept at 45ºC. Two sterilized solutions were added to this medium: 10 mL solution A containing potassium ferricyanide (10% w/v) and 10 mL of solution B containing ferric citrate (1g) and sodium citrate (1g) in 40 mL distilled water. Overnight LAB cultures grown in MRS broth at 37ºC were spotted (10 µL) on KMK agar plates and incubated for 48h at 37ºC. *Lb. plantarum* ATCC 8014 was used as a positive control, while *Lb. fermentum* ATCC 9338 was used as negative control. KMK medium allows distinction between citrate-fermenting colonies (that become blue) and non-fermenting (white) colonies.

Diacetyl production. For diacetyl production screening, LAB strains were inoculated (1% v/v) in 2 mL UHT milk 0.1 % fat and incubated for 24h at 37ºC. The Vosges-Proskauer (VP) reaction was performed - 0.5 mL of a solution α-naphthol (1% w/v) and KOH (16% w/v) were added to the cell suspension. The tubes were stirred and incubated for 10-15 minutes at 37ºC. Diacetyl generation was indicated by the formation of a red ring at the top of the tubes (Franciosi, 2009, *apud* FARAHANI & al., [32]). *Escherichia coli* ATCC 10536 was the negative control for the VP reaction, and *Listeria monocytogenes* ATCC 1911 was the positive control.

Proteolytic activity. For the determination of proteolytic activity, the tested LAB strains were grown on MRS agar plates for 24-48h at 37ºC. A loop (1-2 colonies) of each bacterial culture was spotted on skimmed milk agar plates (SMA) and incubated for 4-7 days at the optimal growth temperature. The SMA medium was composed of 28 g L^{-1} skimmed milk powder, 5 g L^{-1} casein enzymic hydrolysate, 2.5 g L^{-1} yeast extract, 1 g L^{-1} glucose and 15 g L^{-1} agar, pH = 7 (PAILIN & al., [33]). Proteolytic activity was indicated by the occurrence of a clear zone surrounding the colonies, the radius (mm) of which was measured.

Lipolytic activity. For the evaluation of their lipolytic activity, LAB strains were grown on MRS agar plates for 24-48h at 37ºC. A loop (1-2 colonies) of each culture was placed on tributyrin/Tween 80 agar medium (6.7 g L^{-1} YNB with amino acids, 5 g L^{-1} $(NH_4)_2SO_4$, agar 22 g L^{-1}, pH 6.8, with 25 mL of Ty solution – tributyrin 20%, Tween 80 0.5%' – added after sterilization) and he plates were incubated for 4-7 days at 37ºC (CORBU & al., [34]). Lipolytic activity was detected by the presence of a clear halo around the colonies.

Statistical analysis. All screening tests obtained were expressed as percentage charts and calculated with the Excel program in the Microsoft Office 2016 package.

Results and Discussion

Acidification potential and production of organic acids

The acidification potential, due to the production of organic acids, is an important feature in the production of fermented foods, not only for its influence on the organoleptic properties of the end-product, but also for preventing the growth of unwanted (pathogenic or spoiling) microbial strains (SALVUCCI & al., [35]). A rapid decrease of the pH is also essential for the coagulation of milk, the fast acidifying strains being important candidates for dairy fermentation processes as primary starter cultures; still poor acidification strains can be used as adjunct cultures depending on their other properties. After 6h of incubation, no significant modification in the pH range was observed. After 24 h of incubation it resulted that 3% (4 strains) of the tested strains were able to lower the pH of the growth medium to 3.5 and 55% (71 strains) of them had the capacity to acidify milk to a pH range of 4.5-4.0. 42% (55 strains) determined only a slight acidification (Figure 1).

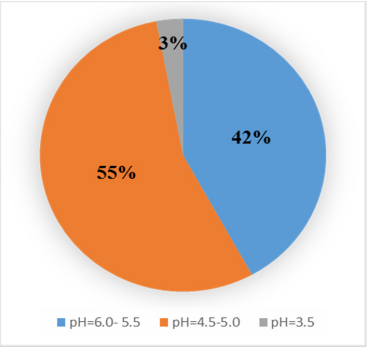

Figure 1. Acidification potential of newly isolated lactic acid bacteria strains after 24h of incubation in milk (Chart generated with the Excel program in Microsoft Office 2016 package).

About 60% of the analyzed LAB strains formed a clear zone surrounding the colonies on MRS+$CaCO_3$ medium. 23 strains formed a clear zone larger than 20 mm. A larger diameter of the clear zone can be correlated with production of a higher amount of organic acids, but it is known that acid production can be affected by temperature and it is probable that the greatest acid production occurs at another temperature than the optimal growth one (VUKASINOVIC & al., [36]), which remains to be studied for the best producers identified by the initial screening.

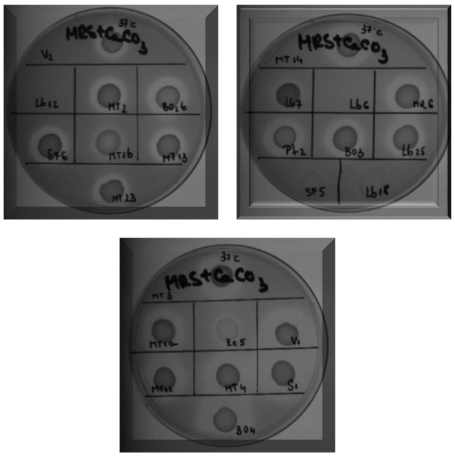

Figure 2. Production of organic acids – clear zones surrounding the colonies on MRS+$CaCO_3$ medium

Exopolysaccharide (EPS) assay

Exopolysaccharides improve the viscosity, texture and flavor of fermented products acting as natural biothickeners, gelling agents or physical stabilizers (FLOROU-PANERI & al., [14]). The EPS assay indicated that some of the LAB strains evaluated formed different types of colonies on MRS-sucrose medium, which corelates with the production of various amounts of EPSs. About 22% (28 strains) of the analyzed LAB strains formed mucoid colonies, while 5% (7 strains) formed ropy colonies on MRS-sucrose agar plates (Figure 3), showing their ability to produce high amounts of EPS – 35 strains were selected for further more detailed studies.

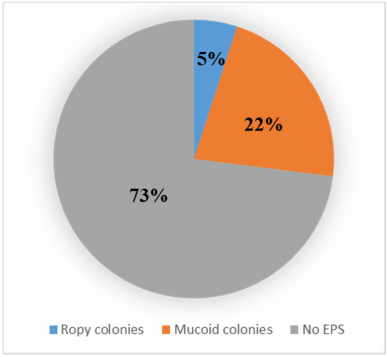

Figure 3. EPS production by the analyzed lactic acid bacteria strains, after 24h of incubation (Chart generated with the Excel program in Microsoft Office 2016 package).

Citrate-fermenting capacity

Citrate metabolism has a key role in lactic acid bacteria food fermentations due to the resulting compounds, such as diacetyl and acetaldehyde that have distinct aroma properties (HUGENHOLTZ, [37]). The citrate metabolism test revealed that 84 % of evaluated LAB strains had the capacity to ferment citrate, as indicated by the appearance of blue colonies on KMK medium agar plates (Figure 4).

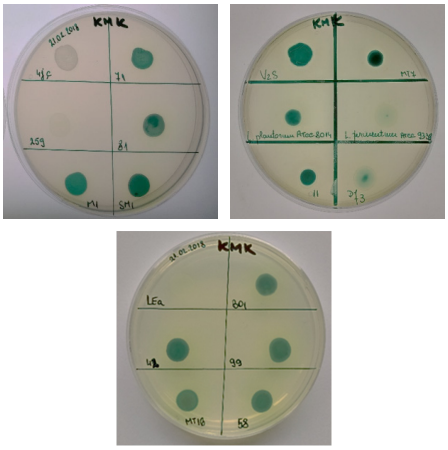

Figure 4. Citrate fermentation on KMK medium – blue colonies = positive (citrate- fermenting); white colonies = negative.

Diacetyl production

The diacetyl production was correlated with the capacity to metabolize citrate since this compound is generated as a final metabolite in the citrate fermentation pathway by some LAB strains. Diacetyl has great technological importance in food industry processes because it contributes to the development of products' flavor. In low concentrations, it is essential in many dairy products, such as butter, fresh cheese and buttermilk (while in wine and beer its presence is detrimental) (HUGENHOLTZ & al., [38]). About 81% of analyzed LAB strains produced diacetyl (Figure 5), different levels of diacetyl production (high, medium and low) being detected; most of them yielded high levels of diacetyl, associated with the apparition of a thick (more than 5 mm) red ring at the top of the tubes.

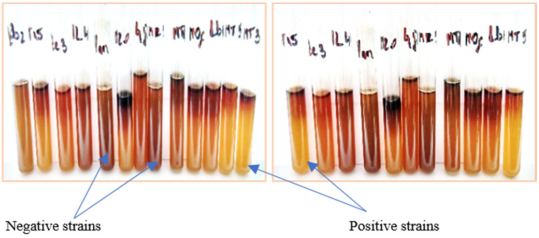

Figure 5. Diacetyl generation (Voges-Proskauer reaction), as indicated by the formation of a red ring at the top of the tubes.

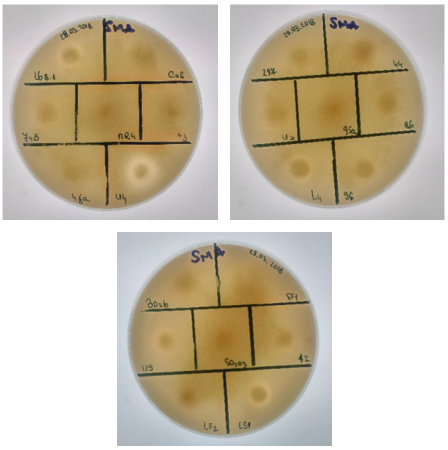

Figure 6. Proteolytic activity as proved by the occurrence of a clear zone surrounding the colonies on SMA plates, after 7 days of incubation

Proteolytic activity

LAB proteolytic activity yields various metabolites that contribute to the development of texture of the products and also to the specific aroma (by generation of certain alcohols, aldehydes, acids, esters, and sulfur compounds) (SAVIJOKI & al., [39]). About 12 % of the tested strains were able to degrade casein in plate assays as indicated by the formation of a clear halo surrounding the colonies, after 7 days of incubation (Figure 6). Good proteolytic activity was observed for 5 strains when tested on skimmed milk agar plates, the clear zones surrounding the colonies being larger than 10 mm.

Lipolytic activity

Lipases are important in the product flavour and texture development. LAB are known as weak producers of lipases and it is generally considered that the lipolysis in fermented foods is mainly due to other strains that are present in starters; still, some LAB strains were recently found to exhibit significant lipolytic activity (DİNÇER and KIVANÇ, [40]). In our case, out of the 130 LAB strains screened 15% were positive, showing clear zones on the agar plates (Figure 7). Seven of the positive LAB isolates had the diameter of the halo surrounding the colony larger than 5 mm after 7 days of optimal temperature incubation, which is associated with a higher lipolytic activity.

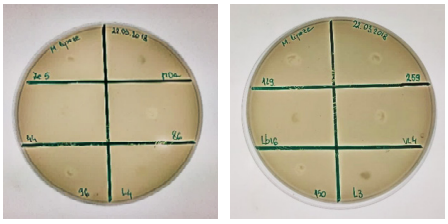

Figure 7. Lipolytic activity of lactic acid bacteria strains, as demonstrated by the clear zones surrounding the colonies on tributyrin/Tween80 medium, after 7 days of incubation.

Conclusions

The local and traditional fermented foods and beverages of Romania represent a large pool of LAB strains. 130 strains isolated from fermented cereals (rye, wheat), vegetables and roots (cucumbers, tomatoes, cabbage, carrots, beetroot, European elder juice, apple juice, orange juice, grape must), and milk products (yogurt, kefir, cheese, sour cream) locally produced in Romania were tested for their biotechnologically relevant properties.

About 60% of the strains showed a significant acidification potential, which is an important feature in the production of fermented foods, both by modelling the organoleptic properties of the end-product, and for preventing the growth of pathogenic and spoiling microorganisms. About 22% of the strains formed mucoid colonies and 5% formed ropy colonies on an adequate medium, showing their ability to produce high amounts of exopolysaccharides that are compounds of importance in the food industry, as they improve the texture and flavour of the

products, and also in the pharmaceuticals industry. About 84% of the evaluated LAB strains had the capacity to ferment citrate, an ability that serves the formation of the aroma compounds diacetyl and acetaldehyde – 81% of the strains were further shown to specifically produce diacetyl. About 12% of the tested strains were able to degrade casein, with five particular strains showing higher proteolytic abilities, such properties of the LAB being known for their contribution to flavour development by generation of certain alcohols, aldehydes, acids, esters, and sulfur compounds. Finally, 15% of the studied strains exhibited lipolytic activity – seven of them manifesting higher lipolytic capacities – a trait that is important for the development of the specific textures and aromas of fermented foods, even though it is not especially strongly manifested in LAB.

Based on the above-mentioned results concerning their functional properties regarded either isolated or combined, 50 newly isolated lactic acid bacteria strains were selected in view of further studies related to potential industrial applications.

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