Highlighting the Antimicrobial Activity of Organic Compounds Isolated from Some Strains of Lactic Acid Bacteria

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One of the beneficial features exerted by the lactic acid bacteria is represented by their ability to produce a large variety of compounds with antimicrobial activity against some strains of bacteria, yeasts and fungi. Among these compounds we encounter both metabolites acting as inhibitors of microbial growth and metabolites with microbicidal effect. The primary objective of this study was to determine the antimicrobial activity of some lactic acid bacteria strains against pathogenic or potentially pathogenic bacterial strains, as well as the correlation of antimicrobial activity with the biosynthesis of organic compounds. The results achieved showed that two strains secrete bacteriocins and organic acids which have antimicrobial activity especially against pathogenic strains belonging to Listeria (L.) monocytogenes and Bacillus (B.) cereus. Bacteriocins produced by the two tested strains belonging to Lactobacillus (L.) hamsteri and Lactococcus (L.) lactis species produced inhibition areas of the tested pathogenic strains, the most pronounced antimicrobial activity was recorded with the L. lactis strain.

Keywords: lactic bacteria, antimicrobial, organic compounds

Human gastrointestinal tract is an environment rich in different microbial species, to the level to which we can find around 800-1.000 different bacterial species and 7.000 strains [1]. As we age, the gut microbiota becomes more complex, being influenced by lifestyle and diet. A main focus of current research is to understand the function and contribution of the human gut microbiota to the host. It has become acknowlegded that the interaction between normal microbiota and the human mucosa is essential for proper intestinal function [2]. Lately, studies on selected strains of lactic acid bacteria probiotic effect have gained momentum. One of the main ecological niches specific to lactic acid bacteria is represented by the foods fermented through traditional methods. Some studies indicated consumption of *Lactobacillus (L.) acidophilus* by healthy adults seemed to lead to altered gene expression network that stimulates and regulates immune responses, hormonal regulation of tissue growth and development, and water and ion homeostasis [3]. Naturally fermented dairy products are an important component in the diet of the population in Romania, especially for the rural population [4].

Fermented dairy food products have been analyzed and it has been pointed out that in addition to the nutritional benefits, they positively influence the physiology of the human body. Dairy drinks (fermented or unfermented) are considered foods out of which can be isolated strains of lactic acid bacteria with probiotic potential. Probiotic *Lactobacillus* strains enhance the integrity of the intestinal barrier, which may result in maintenance of immune tolerance, decreased translocation of bacteria across the intestinal mucosa, and disease phenotypes as well as gastrointestinal infections, irritable bowel syndrome and inflammatory bowel disease [5] . In the process of fermentation natural lactic acid, acetic acid and citric acids are obtained which are commonly used in the food industry to improve the organoleptic properties of several consumer goods [6,7]. One of the beneficial characteristics exerted by the lactic acid bacteria is represented by their ability to produce a large variety of compounds with antimicrobial action against some strains of bacteria, yeasts and fungi. Among these compounds, there are both metabolites with role of of microbial growth inhibitors, as well as metabolites having a microbicide effect, such as, for example, organic acids (lactic acid, fenillactic and hydroxyphenyl acid, acetic acid, propionic acid, etc.), bacteriocins and bacteriocin-like compounds, hydrogen peroxide, reuterin. Bacteriocins are antimicrobial proteins or oligopeptides synthesized by a number of Gram-positive and Gram-negative bacterial strains of and, generally, have a much narrower spectrum of activity than synthetic antibiotics. Some studies have been conducted for the isolation and characterization of bacteriocins in terms of chemical properties [8]. Bacteriocins have an important practical application in the preservation of foods but also in the prevention of bacterial infections. Most of the bacteriocins produced by lactic acid bacteria are thermostable, thereby maintaining their activity even after the food heating preservation processes. The mechanisms of action of bacteriocins are diverse and complex due to the particular chemical structure, they operate in most cases on the cellular membrane by the formation of pores or at the level of essential processes of living cells (transcription, translation, replication, biosynthesis of cell wall components) [9]. The bacteriocin gains entry into the target cell by recognizing specific cell surface receptors and than kills the cell by formating ion-permeable channels into the cytoplasmic membrane, by nonspecific cleavage of 16s RNA, or by cell lysis resulting from inhibition of peptidoglycan synthesis. However,

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All authors have equal contribution to the study and the publication

research has shown that there are a variety of ways of action; a single bacteriocin possesses more than one way of action to atack the target bacteria [10].

Some species of lactic acid bacteria produce antimicrobial compounds such as ethanol, formic acid, fatty acids, hydrogen peroxide, diacetyl, acetaldehyde, D-isomers of amino acids. In recent years, species of lactic acid bacteria isolated from products obtained by spontaneous fermentation which produce bacteriocins have been extensively studied because of their potential of being used as safe additives for food preservation [11]. Previous research conducted in this area have shown the antimicrobial effect of some strains of lactic acid bacteria and nonsteroidal antiinflamatory drugs against certain strains of the species *Candida (C.) albicans* [12] and some pathogenic bacterial strains (*Salmonella (S.) enteridis*, *Escherichia (E.) coli*, *Bacillus (B.) cereus*, *Shigella (S.) flexerii*). It has also been observed an increased sensitivity to fluconazole of the yeast strain in the presence of lactic acid bacteria [13]. We analyzed the antimicrobial activity of some strains of lactic acid bacteria belonging to the genera *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Lactococcus* against Gram-positive and Gram-negative bacterial species. The results have showed that most strains of selected lactic acid bacteria produce active compounds on solid environment, which had antagonistic properties against *S. typhimurium and E. coli* strains [14].

The primary objective of this study was to determine the antimicrobial activity of certain strains of lactic acid bacteria against pathogenic bacterial species as well as highlighting the nature of organic compounds action against analyzed pathogenic species.

Results and discussions

Material and methods

Strains and culture medium

There were studied a total of six new strains of lactic acid bacteria isolated from fecal samples from babies fed on breast milk and traditional fermented products.

In order to determine the antimicrobial activity of lactic bacteria strains there were used pathogenic or potentially pathogenic microbial strains. For cultivation of lactic acid bacteria strains a -Man Rogosa-Sharpe broth was used (MRS) at *p*H = 6.5. The strains were cultured for 18-24 h at 37°C.

Pathogenic microbial strains were cultured in Brain Heart Infusion (BHI g/ L: Calf Brain Infusion 7.7 ; beef heart infusion 9.8; peptone 10; dextrose 2; sodium chloride 5; phosphate 2.5; *p*H 7.4) and Luria-Bertani respectively (LB g/ L: tryptone 10, yeast extract 5, sodium chloride 10) at 37°C for 18-24 h.

Determining antimicrobial activity

In order to determine the antimicrobial activity of lactic acid bacteria strains full culture was tested, supernatant (unadjusted *p*H) and the supernatant adjusted to *p*H 6.5 by using several methods of micro-organisms cultivation. 100µL of fresh culture ($D.O._{600nm}$ 0.4-0.6) of each pathogen were dispersed on plates with BHI or LB medium (with agar 15%). After drying the plates, 10µL of lactic acid bacteria culture with a density of $D.O._{600nm}$ 0.8 was added in spot. The plates were incubated 24-48 h at 37°C. After incubation, strains of lactic acid bacteria with antimicrobial activity showed a clear area around the culture spot as a result of inhibiting the pathogen microorganism growth.

Strains of lactic acid bacteria with antimicrobial activity were grown in MRS liquid environment for 24 h at 37 ° C without stirring. After centrifugation for 15 min at 12.000 rpm, the filtered supernatant was tested (*p*H adjusted to 6.5 and the non-adjusted) compared to susceptible pathogenic microorganisms in order to determine the antimicrobial activity and its correlation with the biosynthesis of organic acids.

Strains for which the supernatant having *p*H 6.5 showed antimicrobial activity were grown in 50 mL MRS environment for 18 h at 37ÚC. The supernatant obtained after centrifugation for 15 min. at 12.000 rpm was filtered and the *p*H adjusted with 40% NaOH solution to a value of 6.5.

After adjustment of the pH, $(NH_4)_2SO_4$ was slowly added up to saturation of 40% (243g/L). The solution was saturated with ammonium sulfate overnight at 4°C at 100 rpm (gentle shaking). The next day the solution was centrifuged for 15 min at 12.000 rpm at 4°C, and the precipitate (brown colour) re-suspended in 500 µL sterile distilled water. In 50 µL of the protein extract Proteinase K (Sigma) was added in final concentration of 1 mg/mL and the samples were incubated for 1h at 37°C. Antimicrobial activity of protein extracts treated and untreated with proteinase K was determined by diffusimetric method mentioned above. For strains of the genera *Bacillus* and *Listeria* were prepared BHI plates (1.5% agar) and LB medium (1.5% agar). A 100 mL of culture of the indicator strains having DO_{600nm} ranging between 0.4 and 0.5 were used to inoculate 3.5 mL semi-solid BHI medium (0.7% agar), and it was poured into Petri plates previously prepared. After the medium solidified on its surface there were added 10 mL of samples analyzed in the form of spots. The plates were incubated for 24 h at 37 and 30°C respectively. After incubation, the indicator strains develop in the layer and the presence of antimicrobial compounds in the supernatant was evidenced by the occurrence of growth inhibition areas (clear areas) around the spots or folds.

Results and discussions

Determining antimicrobial activity by diffusimetric method

After determining the antimicrobial activity by diffusimetric method it has been found that most strains of lactic acid bacteria showed antimicrobial activity against pathogenic strains analyzed, especially against strains of the species *Stahylococcus (S.) epidermidis*, *S. aureus*, *L. monocytogenes* and *E. coli*. The results obtained showed that the spectrum of inhibition was different for the strains analyzed. In figure 1 (A) it can be noticed that the six strains of lactic acid bacteria strains presented antimicrobial activity against pathogenic analyzed strains. Table 1 shows the results regarding the antimicrobial activity of selected lactic acid bacteria by measuring the inhibition area. The positive result was considered a diameter of inhibition area with size greater than 1 mm. The results we obtained showed that these strains analyzed E2.3, E4.2 and F2a have developed considerable areas of inhibition against most pathogenic strains tested.

Staphylococci are the most commonly encountered species of bacteria that colonize the skin and mucous membranes in humans. In particular, *S. epidermidis* is the most frequently isolated species in the human epithelium that colonizes predominantly the armpit, head and nostrils [15-17]. Drug treatment for infections caused by this species is complicated because of antibiotic resistance genes and biofilm formation, which show intrinsic resistance to antibiotics and host defense mechanisms [18]. An alternative to the classic treatment in order to combat bacterial infections is the bacterial interference, *bacteriotherapy* in which commensal bacteria are used in order to prevent colonization of the host by various pathogenic microbial species.

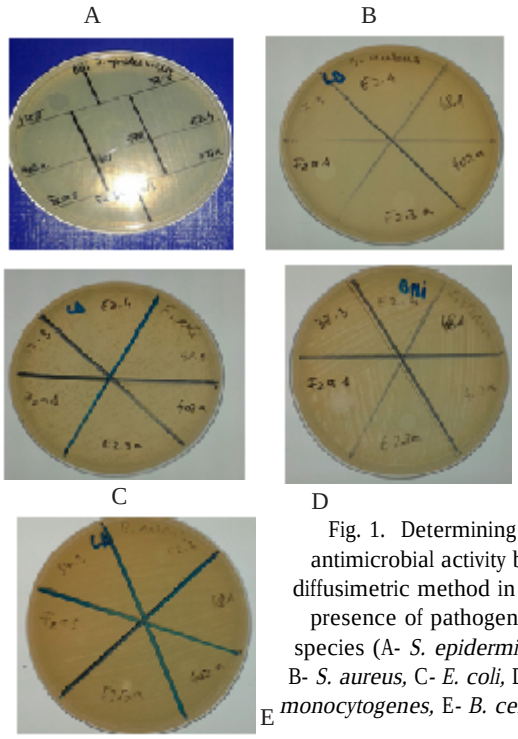

Fig. 1. Determining antimicrobial activity by diffusimetric method in the presence of pathogenic species (A- *S. epidermidis*, B- *S. aureus*, C- *E. coli*, D- *L. monocytogenes*, E- *B. cereus*)

Tests conducted have shown an increased antimicrobial activity of *L. hamsteri* E2.3 strain on most of the species tested pathogenic belonging to *S. aureus* , *L. monocytogenes* and *E. coli* species. They are consistent with the results obtained by other researchers on the strains of *S. aureus* and *E. coli* species [19]. Moreover, the vast majority of species belonging to the genus *Lactobacillus* exhibited high antimicrobial activity against *S. aureus* strains [20]. The *L. plantarum* E4.2 strain inhibited growth of *S. aureus* strains (diameter of 5 mm), *L. monocytogenes* (5 mm) and *E. coli* (4 mm) (table 1). Strains within *L. monocytogenes* species can lead to an infection called listeriosis. This is an intracellular pathogen, which penetrates into the cytosol and uses the lipoic acid of the host in order to replicate. In the US 1.600 people get sick and 260 die annually because of this, according to Center for Disease Control and Prevention [21]. Human listeriosis is an opportunistic infection that affects most commonly people with severe intercurrent diseases, the elderly, pregnant women, fetuses, newborns and adults with weakened immune systems. Control of this pathogen remains a challenge for the food industry as it is ubiquitous in nature and has the ability to grow at low temperatures and survive in hostile environments. At European level, listeriosis is the main pathology within food poisoning cases, followed by salmonellosis [22].

The *L. lactis* F2a strain showed, in the tests we conducted, an inhibitory activity of pathogenic strains growth, as evidenced by the appearance of a diameter of 7 mm for *S. aureus* strain and 6 mm for *L. monocytogenes* strain. Isolated bacteria from fermented milk shows antimicrobial activity which is due to production of organic acids (lactic acid, acetic acid) and not due to the biosynthesis of bacteriocins.

Two of the strains analyzed by us, *L. hamsteri* E2.3 and *L. lactis* F2a developed a high area of inhibition against pathogenic species *S. aureus* and *L. monocytogenes* whereas the strain *L. hamsteri* E2.3 showed a wider spectrum of action having an inhibitory activity against strains belonging to *E. coli*, *S. epidemidis* and *B. cereus* species.

Determining compounds with antimicrobial action released by lactic bacteria

Diffusimetric method in agar was used to determine the antimicrobial activity of a number of two strains of

Pathogenic strains analyzed	Lactic acid bacteria strains analyzed	Inhibition area (mm)
S. aureus	F2a	7
	E2.3	7
	E4.2	5
	402	+/-5
S. epidermidis	E2.3	7
	428 ST	7
	E4.2	5
L. monocytogenes	F2a	6
	E2.3	6
	E4.2	5
E. coli	E2.3	5
	E4.2	+/-4
	48.1	+/-6
B. cereus	E3	+/-2

Table 1
THE INHIBITION AREA OF LACTIC BACTERIA STRAINS DEVELOPED BY ANALYZED BY US(+/- LESS CLEAR AREA)

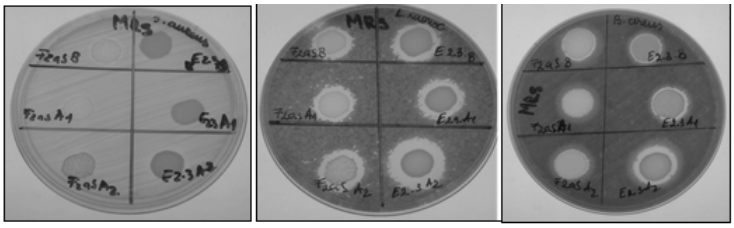

Fig. 2. Antimicrobial activity of *L. hamsteri* E2.3 and *L. lactis* F2a strains analyzed by us for the production of bacteriocin and/or organic acid from *p*H=6-6.5) (B - bacteriocins, A_1-to non-adjusted pH and A_2 to the adjusted *p*H)

Pathogenic strains analyzed	Lactic acid bacteria strains analyzed for bacteriocin or acid organic	Inhibition area (mm)
S. aureus	E2.3 B	-
	E2.3A_1	-
	E2.3A_2	+/- 14
	F2a B	+/- 11
	F2a A_1	-
	F2a A_2	-
L. monocytogenes	E2.3 B	+16
	E2.3A1	+15
	E2.3A2	+18
	F2a B	+18
	F2a A_1	+14
	F2a A_2	+15
B. cereus	E2.3 B	+11
	E2.3A_1	+13
	E2.3A_2	+15
	F2a B	+13
	F2a A_1	+11
	F2a A_2	+12

Table 2
AREA OF INHIBITION DEVELOPED BY THE TWO STRAINS OF LACTIC ACID BACTERIA ANALYZED E2.3 AND F2a FOR IDENTIFICATION OF BACTERIOCINS AND/OR OF ORGANIC ACIDS ACTIVITY (+, ANTIMICROBIAL ACTIVITY AREA; +/-, AN AREA WITH UNCERTAIN /UNCLEAR ACTIVITY, B-BACTERIOCINS, A_1-TO NON-ADJUSTED *p*H AND A_2 TO THE ADJUSTED *p*H)

lactic acid bacteria isolated from different sources. Their antibacterial properties were tested against pathogenic strains: *L. moncytogenes*, *S. aureus*, *E. coli*, *S. epidermis* and *B. cereus* (fig. 2). Table 2 presents the results regarding the antimicrobial activity of isolated lactic acid bacteria analyzed. A larger diameter of 1 mm was considered a positive result. The results we obtained within this study indicate that strains *L. hamsteri* E2.3 and *L. lactis* F2a had increased antibacterial properties against all strains tested, except for *S. aureus* strains. The most increased antibacterial action was observed on *L. monocytogenes* strain, with an area of inhibition of 16 mm produced by the *L. hamsteri* E2.3 bacteriocin producing strain, resulting certified by the diameter of inhibition area produced in the case of adjusted *p*H (18 mm). A similar result was obtained in our tests for the bacteriocins produced by *L. lactis* F2a strain which showed a 18 mm area of inhibition against pathogenic strain of *L. monocytogenes* and 15 mm after adjusting *p*H values. In case of two strains of lactic acid bacteria, the antimicrobial activity was correlated with the production of bacteriocins. Bacteriocin produced by *L. lactis* F2a strain showed the highest activity.

Regarding the occurrence of the antimicrobial activity manifested by the presence of organic acids produced by the two strains of lactic acid bacteria isolated by us (A_1, with an acid pH, not adjusted) it was observed that both strains secrete organic acids (in addition to the bacteriocin); their activity was increased towards the pathogenic strain of *L. monocytogenes*, 15 mm for *L. hamsteri* E2.3 strain and 14 mm for *L. lactis* F2a strain. The presence of the *L. hamsteri* E2.3 strain in culture medium with *B. cereus* species has led to the development of areas of inhibition of 13 mm and *L. lactis* F2a strain resulted in the appearance of an area of inhibition less than 11 mm (fig. 2 C). It can be noticed that the two studied strains of lactic acid bacteria *L. lactis* F2a and *L. hamsteri* E2.3 showed antimicrobial activity on Gram-positive strains represented by food pathogens, *L. monocytogenes* and *B. cereus*.

Some of the most important and known compounds involved in the antimicrobial activity of lactic acid bacteria are organic acids. They are able to inhibit the growth of pathogenic or potentially pathogenic bacteria, because they lead to decreased *p*H, thus providing an acidic environment unfavorable to the development of pathogenic microorganisms. The mechanism of action of lactic acid is by diffusing in the cell of the undissociated form where its dissociation occurs and the generation of hydrogen ions which increase cytoplasmic acidity level, resulting in disruption of the cell metabolic activity. Organic acids have a broad spectrum of action, inhibiting both Gram-negative and Gram-positive bacterial strains, as well as a number of yeasts and fungi strains. Bacteriocins are classified into four groups based on their structure, molecular mass, thermostability, enzymatic susceptibility and mechanism of action [23]. These compounds have an important practical application in food preservation as well as in prevention of bacterial infections [24]. They have a limited spectrum of inhibition acting especially on Gram-positive bacteria, but many bacteriocins produced by lactic acid bacteria are active against food pathogens such as *B. cereus*, *Clostridium (C.) botulinum*, *C. perfringens*, *L. monocytogenes*, *S. aureus*, etc. [25]. Some bacteriocins such as nisin, mersacidin, mutacin 1140, and lacticin 3147 are reported to be active against drug-resistant MRSA and VRE which makes them an attractive option as possible therapeutic agents [26]. Many of bacteriocins produced by lactic acid bacteria are thermostable, so they retain their activity after thermal food preservation processes [27, 28].

Conclusions

As regards the determination of antimicrobial activity by diffusimetric method we found that most strains of lactic

acid bacteria we tested have presented antimicrobial effect against pathogenic bacterial strains analyzed, especially against strains of the species *S. aureus*, *S. epidermidis* , *L. monocytogenes* and *E. coli*. We have also conducted experiments in order to emphasize the production of bacteriocins and/or organic acids (compounds with antimicrobial role) on some newly isolated lactic acid bacteria strains..

Our results revealed that two of the analyzed strains secret, besides organic acids, bacteriocins that act especially against pathogenic *L. monocytogenes* and *B. cereus* strains. Bacteriocins produced by the two strains tested *L. lactis* F2a and *L. hamsteri* E2.3 have produced different areas of inhibition, the most pronounced activity being presented by the bactericin released by *L. lactis* F2a strain.

Acknowledgments: This work has been supported by project: Executive Agency for Higher Education, Research, Development and Innovation Funding (UEFISCDI) project PN-II-PT-PCCA-2011-3.1-0969 (PROLAB 77/2012).

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Manuscript received: 30.03.3016