

Antagonistic action of *Lactobacillus* spp. against *Staphylococcus aureus* in cheese from Mompox – Colombia

Acción antagonística de *Lactobacillus* spp. frente a *Staphylococcus aureus* encontrados en queso de capa de Mompox – Colombia

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Abstract. In the food industry, food preservation techniques that do not use chemical products are becoming more common. Therefore, the aim of this research was to evaluate the antagonistic activity (antibiosis) of lactic-acid bacterial strains against pathogenic microorganisms. Lactic-acid bacterial strains were isolated from layered cheese and a commercial product (yogurt); and the same was done with pathogenic bacteria solely from layered cheese. The lactic-acid bacterial strains were identified as species from the Lactobacilli family, while the pathogenic bacteria from layered cheese were identified as Micrococcaceae family species (*Staphylococcus aureus*). Subsequently, in the same culture medium, bacteria of each species were sowed in order to determine the inhibitory activity ability of the Lactic Acid Bacteria (BAL) As a result, the highly antagonistic activity of the Lactobacilli (inhibition halos were larger than 0.5 centimeters in diameter) against isolated pathogenic microorganisms was demonstrated.

Key words: Antibiosis, bacteriocins, dairy products, *Lactobacillus*, *Staphylococcus aureus*, (Mesh Database).

Resumen. En la industria alimentaria, cada vez es más frecuente el uso de técnicas que permiten la conservación de alimentos, sin el uso de productos químicos tradicionales. Por lo tanto, el objetivo de este trabajo fue evaluar la actividad antagonista (antibiosis) de las cepas de bacterias ácido lácticas contra microorganismos patógenos. Las cepas de bacterias ácido lácticas fueron aisladas de queso de capa y un producto comercial (yogurt), y lo mismo se hizo con las bacterias patógenas, las cuales fueron aislada del queso capa. Las cepas de bacterias ácido lácticas se identificaron como especies de la familia lactobacillaceae, mientras que las bacterias patógenas del queso de capa se identificaron como especies de la familia Micrococcaceae (*Staphylococcus aureus*). Posteriormente, las bacterias de cada especie fueron dispuestas con el fin de determinar la inhibición del crecimiento de las bacterias patógenas por Bacterias Ácido Lácticas (BAL). Estos resultados mostraron que los lactobacilos inhibieron el crecimiento de *Staphylococcus aureus*, evidenciándose la actividad antagonística (halos de inhibición más grandes de 0,5 centímetros de diámetro) contra microorganismos patógenos aislados de este productos.

Palabras clave: Antibiosis, bacteriocinas, productos lácteos, *Lactobacillus*, *Staphylococcus aureus*, (Decs Bireme).

Cheese is a good culture medium for microorganism growth because of its high water content, its pH, and the variety of compounds it contains (Settanni *et al.*, 2012). One of the higher proportion ingredients in cheese is cow milk, which, due to rudimentary milking processes and transport, gains microbiota that include beneficial, saprophytic and pathogenic microorganisms (Bernardeau *et al.*, 2008).

Traditionally, the pathogenic bacteria of most concern to food microbiologists are, among others: *Staphylococcus aureus*, *Salmonella* spp., and *Clostridium botulinum*. Nowadays, *Staphylococcus aureus* is one of the bacteria frequently linked to food poisoning outbreaks (Rodriguez *et al.*, 2009) due to its wide distribution in the environment and its ability to produce toxins (Feutry *et al.*, 2012).

Nevertheless, in the last few years, certain microorganisms have gained relevance as food poisoning causative agents, for example: *Listeria monocytogenes*, *Bacillus cereus*, *Aeromonas hydrophila*, *Yersinia enterocolitica*, *Escherichia coli* verotoxigenic (ECVT), and *Campylobacter jejuni* (Ladero *et al.*, 2010; Carraro *et al.*, 2011).

Likewise, as a consequence of the abuse of antibiotics in medicine and farm animals, there has been a significant increase in multi-antibiotic resistant food pathogens (Call *et al.*, 2008; Walsh and Fanning, 2008).

Currently, Lactic-acid bacteria present a substantial biotechnological potential due to its presence in several fermentative processes for human and animal food production, specifically those related with dairy products,

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vegetables, meat, baked goods, alcoholic beverages, and silages (Monteagudo *et al.*, 2011).

These bacteria not only contribute to the development of organoleptic and rheological characteristics in the food, but also generates environments that were only slightly favorable for the development of pathogenic microorganisms due to their marked antagonistic capacity, which inhibits their proliferation in food over any other group of microorganisms that is present in the preliminary material (raw food) or that contaminates the subsequent product (Samelis *et al.*, 2014).

The beneficial effects are caused by the antagonist ability, also known as antibiosis, which is based on organic acids and the production of other inhibitory metabolites. Among these metabolites, it is important to mention oxygen peroxide (H₂O₂) and others that are derived from oxygen metabolism, aromatic compounds (diacetyl, acetaldehyde), dehydrated derivatives of glycerol (reuterin), bacteriolytic enzymes, bacteriocins and so forth (Madureira *et al.*, 2011; Georgalaki *et al.*, 2013).

In bacteriocin inhibitory mechanisms, the pore formation in the cytoplasmic membrane of sensitive cells seems to be a common action mechanism of substances produced by lactic acid bacteria. The structure of these peptides, α -helixes or β -laminates has two sides, an hydrophilic one and an hydrophobic one, which create oligomers that go through the membranes, forming pores; the hydrophobic side places itself close to the lipids of the membrane, while the hydrophilic side goes to the center of the pore (Nissen *et al.*, 2010). This action produces losses in several essential compounds, such as K⁺ ions, ATP and, in some cases, amino acids and small molecules simultaneously, these losses trigger a membrane potential reduction, consumption of energy reserves in cells, and a decrease in the synthesis of DNA, RNA and proteins, which could ultimately lead to cell death (Reder and Bendas, 2011).

The bacteriocins produced by lactic acid bacteria are an innovative tool for the control of pathogens in food products as well as those pathogens that spoil food (Nielsen *et al.*, 2010). Likewise, several strains can contribute to the preservation of fermented food through the production of bacteriocins (Castro *et al.*, 2011). Studies on bacteriocins have increased during recent decades, including on bacteriocin use, or the microorganisms that produce them, as a natural preservative (Tolinački *et al.*, 2012).

The objective of the present study, was to investigate the possible antagonism between BAL bacteria and

pathogenic bacteria isolated from artisan layered cheese manufactured in Mompox, Colombia.

MATERIALS AND METHODS

Sampling and initial isolation of microorganisms. The layered cheese samples were taken in different places in Mompox. They were taken in triplicate and collected at a sufficient quantity and from sterile conditions. The samples were transported in Styrofoam boxes with ice to the Universidad of Cartagena Microbiology Laboratory (Engineering Program-Pilot Plants). The samples were processed under sterile conditions in DIES SEGURA bs 100 flow cabinets, and the incubation was performed in an incubator. Esco, IFA -54-8 USA.

Lactobacilli were isolated from a yogurt commercialized in Cartagena. This yogurt contained *L. acidophilus* and *L. casei*. In order to isolate and identify these microorganisms, Man-Rogosa Sharp-agar was used (MRS) while Brain Heart Infusion agar was chosen for the *S. aureus* isolation (Pereira *et al.*, 2011).

Staphylococcus aureus isolation and identification was done using the Imadi, Tavakoli and Naderi method (Imani *et al.*, 2010). 10 g from the sample were weighed in sterilized bottles with 90 mL BHI broth (Brain Heart Infusion), homogenized and incubated for 37°C/24 h. After that, sowing was done in Petri dishes with salt mannitol agar, a *S. aureus* selective culture medium (Onoue *et al.*, 2002). The dishes were incubated for 37°C/24 h. Strain confirmation was done with catalase and coagulase tests.

Lactic-acid bacteria isolation from commercial product (yogurt) and layered cheese. The isolation method of Lin was followed (Lin *et al.*, 2006). In order to isolate strains from layered cheese, 10 g were weighed and homogenized with peptoned water 0,1% p/v. With these homogenized samples, sowing was done in Petri dishes using MRS agar, with incubation at 37°C for 48 hours.

Isolation from the commercial product was done with MRS agar, following the same method for layered cheese. After the isolation of the lactic acid bacterial (LAB), the following tests were performed: lactose fermentation, glucose, Gram coloration and catalase.

Layered cheese samples that contained *S. aureus* were obtained during several periods of the year and from different handmade-producers in Mompox. The method

of Todorov was carried out to determine the active bacteriocins in the cheese (Todorov *et al.*, 2011). 5 g of homogenized cheese were taken at 50°C with 10 mL of sterilized HCl 0.02 N and centrifuged at 12000 rpm at 4°C for 20 minutes. The supernatant was neutralized with NaOH 1 N until a pH of 6 was reached and then frozen at 20°C for 24 hours. Afterwards, the neutralized sample was inoculated with *S. aureus* in BHI broth.

The antimicrobial activity was verified by the agar well diffusion technique using a matrix with five little, stainless-steel rods (Abo-Amer, 2007). In each well, 100 µL of extract were dispensed, and each one was sealed with a thin agar layer; then, they were kept at refrigerated temperatures in order to facilitate the agar diffusion. After some time, 15 µL of 6x10⁸ *S. aureus* suspension, according to the McFarland scale, were mixed with 12 mL of 0.8% BHI broth. This mixture was added to the agar with the refrigerated extracts and incubated at 37°C for 48 hours. Antimicrobial activity was evidenced by inhibition halos (Haroun *et al.*, 2011).

Antagonist activity spectrum. The method described by Agg and McGiven in 1971 was used. The agar well diffusion technique was used with fluent MRS agar (15 mL) at 45°C. The little, stainless-steel rods were positioned. Everything was kept at room temperature until the culture medium solidified; at this point, the little, steel rods were removed, leaving the Petri dish with small wells ready for sowing. In the wells, quantities

of 6x10⁶, 1.5x10⁷, 3x10⁷, and 6x10⁷ of the commercial *Lactobacilli* from the essay sample were deposited and an additional layer of MRS agar was added to seal the wells. After solidification, the Petri dishes were incubated for 12 hours at refrigerated temperatures, then 15 µL of 6x10⁸ *S. aureus*, according to the McFarlan scale, isolated from the layered cheese from Mompox, were inoculated in 12 mL of BHI agar thawed at 45°C. Subsequent to the homogenization, the final mixture was added to MRS Rogosa agar-Petri dishes with the commercial *Lactobacilli*, resulting in a double agar layer. A period was allocated for solidification, followed by incubation at 37°C for 24 hours. The inhibition halos were then measured around the wells.

The results are shown as mean ± standar error of the three independent experiments. The significance level for the total coliformes was P<0.05.

RESULTS AND DISCUSSION

Staphylococcus aureus identification and count.

The isolation was done using salt mannitol agar, as can be seen in Table 1. The *S. aureus* identification was positive in all of the analyzed samples. Confirmation was carried out with catalase and coagulase tests, which were also positive for *S. aureus*.

Lactic-acid bacteria isolation from commercial product (yogurt) and cheese.

After isolation with

Table 1. *Staphylococcus* spp. growth and identification.

Sample	Salt mannitol growth	Characteristics	Coagulase	Catalase	Gram staining
M-1	+		+	+	
M-2	+		+	+	
M-3	+		+	+	
M-4	+		+	+	
M-1	+		+	+	
M-2	+	Smooth strains, irregular and yellow colored.	+	+	Positive Gram coccus
M-3	+		+	+	
M-4	+		+	+	
M-1	+		+	+	
M-2	+		+	+	
M-3	+		+	+	
M-4	+		+	+	

MRS agar and M17 agar, a significant growth was found exclusively in the second culture medium, indicating a *Streptococci lactic* presence M17 Agar is

used for isolating and enumerating lactic streptococci in yogurt, cheese starters and other dairy products Difco™ & BBL™ as can be seen in Table 2.

Table 2. Microorganism isolation from layered cheese.

Sample	Rogosa	M-17	Characteristics
M-1	-	+	
M-2	-	+	
M-3	-	+	
M-4	-	+	
M-1	-	+	
M-2	-	+	<i>Small colonies, convex, smooth, opaque and without any pigmentation</i>
M-3	-	+	
M-4	-	+	
M-1	-	+	
M-2	-	+	
M-3	-	+	
M-4	-	+	

Active bacteriocin determination in the cheese.

The evaluation for this substance was negative (Table 3). The determination of Active bacteriocins in the cheese by the agar well diffusion technique did

not show any inhibition halos for *S. aureus*; this is due to the proteic nature of these substances which are inactivated, totally or partially, by proteolytic enzymes (Leonardo *et al.*, 2001; Tuchilus *et al.*, 2007).

Table 3. Bacteriocin determination.

Active bacteriocins	Indicator microorganism	Mc Farland scale	Growth (24 hours)
1	<i>Staphylococcus aureus</i>	6	Yes
2	<i>Staphylococcus aureus</i>	6	Yes

Antagonist activity spectrum. After all the tests with Lactobacilli (wells) and the different strains of *S. aureus* using several microbial densities according to the McFarland scale were done, it was found that the Lactobacilli isolated from the commercial products inhibited growth of the pathogens, as can be seen in Table 4, which can be considered as an antagonistic activity because previous essays showed that this

kind of activity exists when halos are larger than 2 mm, according to Todorov *et al.* (2011).

Inhibition spectrum averages related to *S. aureus* show that 1.5×10^7 was the quantity that produced the most significant inhibition, as compared to the other evaluated densities, as can be seen in Figure 1.

Table 4. Antagonist activity spectrum of the *S. aureus* isolated from the layered cheese from Mompox. P <0.05

Lactic-acid <i>Lactobacillus</i> (Lactobac)	<i>Staphylococcus aureus</i> density	Inhibition diameter
6x10 ⁶	6x10 ⁸ CFU/mL	7.5 mm
		9.0 mm
		6.5 mm
		9.0 mm
1.5x10 ⁷	6x10 ⁸ CFU/mL	5.0 mm
		8.0 mm
		7.5 mm
		6.0 mm
3x10 ⁷	6x10 ⁸ CFU/mL	8.0 mm
		20.0 mm
		7.5 mm
		10.0 mm
6x10 ⁷	6x10 ⁸ CFU/mL	8.0 mm
		15.0 mm
		8.5 mm
		7.5 mm

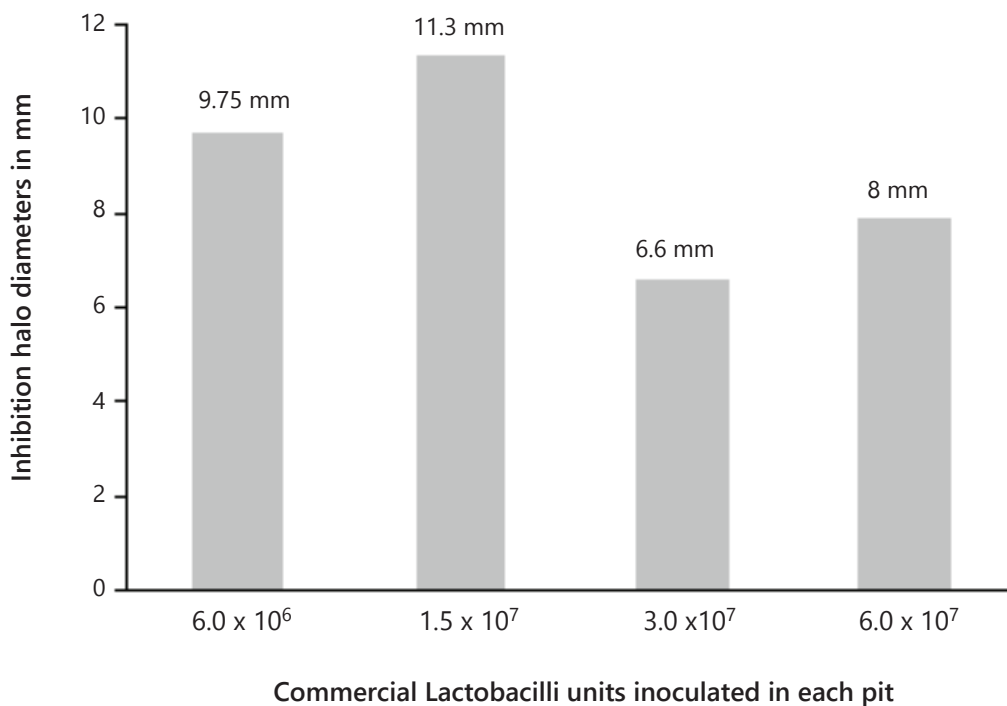


Figure 1. *Staphylococcus aureus* inhibition spectrum.

CONCLUSIONS

The results obtained *in vitro* show that commercial BAL possess biological characteristics compatible with potential use as a probiotics.

The determination of the antimicrobial activity in the layered cheese was negative, as was the Lactobacilli determination, showing that the addition of BAL during processing is important.

The results of the Inhibitory spectrum demonstrate the sensitivity of pathogenic microorganisms, specifically *S. aureus*, to lactic-acid bacteria.

The presence of undesirable microorganisms was confirmed during the cheese production process.

The use of BAL at concentrations equal to 1.5×10^7 had the most relevant inhibitory effect against *S. aureus*.

The use of BAL is considered as other conservation methods which can be an interesting option to diminish the addition of chemical preservatives, providing safe foods naturally preserved.

REFERENCES

- Abo-Amer, A. E. 2007. Molecular characterization of antimicrobial compound produced by *Lactobacillus acidophilus* AA11. *Acta Microbiol Immunol Hung* 54(2): 107-119. doi: 10.1556/AMicr.54.2007.2.2
- Bernardeau, M., J. Vernoux, S. Henri-Dubernet and M. Guéguen 2008. Safety assessment of dairy microorganisms: the *Lactobacillus* genus. *International journal of food microbiology* 126(3): 278-285. doi: 10.1016/j.ijfoodmicro.2007.08.015
- Call, D., M. Davis and A. Sawant (2008). Antimicrobial resistance in beef and dairy cattle production. *Conference of Research Workers in Animal Diseases. Animal Health Research Reviews* 9: 159-167. doi: 10.1017/S1466252308001515
- Carraro, L., M. Maifreni, I. Bartolomeoli, M. E. Martino, E. Novelli, F. Frigo, M. Marino and B. Cardazzo 2011. Comparison of culture-dependent and-independent methods for bacterial community monitoring during Montasio cheese manufacturing. *Research in microbiology* 162(3): 231-239. doi: 10.1016/j.resmic.2011.01.002.
- Castro, M., N. Palavecino, C. Herman, O. Garro and C. Campos 2011. Lactic acid bacteria isolated from artisanal dry sausages: characterization of antibacterial compounds and study of the factors affecting bacteriocin production. *Meat science* 87(4): 321-329. doi: 10.1016/j.meatsci.2010.11.006.
- Feutry, F., M. Oneca, F. Berthier and P. Torre 2012. Biodiversity and growth dynamics of lactic acid bacteria in artisanal PDO Ossau-Iraty cheeses made from raw ewe's milk with different starters. *Food microbiology* 29(1): 33-42. doi: 10.1016/j.fm.2011.08.011
- Georgalaki, M., K. Papadimitriou, R. Anastasiou, B. Pot, G. Van Driessche, B. Devreese and E. Tsakalidou 2013. Macedovicin, the second food-grade lantibiotic produced by *Streptococcus macedonicus* ACA-DC 198. *Food microbiology* 33(1): 124-130. doi: 10.1016/j.fm.2012.09.008
- Haroun, A. A., E. Ahmed and M. A. Abd El-Ghaffar 2011. Preparation and antimicrobial activity of poly (vinyl chloride)/gelatin/montmorillonite biocomposite films. *J Mater Sci Mater Med* 22(11): 2545-2553. doi: 10.1007/s10856-011-4437-x
- Imani Fooladi, A., H. Tavakoli and A. Naderi 2010. Detection of enterotoxigenic *Staphylococcus aureus* isolates in domestic dairy products." *Iran J Microbiol* 2(3): 137-142.
- Ladero, V., M. Fernández, I. Cuesta and M. A. Alvarez 2010. Quantitative detection and identification of tyramine-producing enterococci and lactobacilli in cheese by multiplex qPCR. *Food microbiology* 27(7): 933-939. doi: 10.1016/j.fm.2010.05.026
- Leonardo, M. R., L. A. da Silva, M. T. Filho, K. C. Bonifacio and I. Y. Ito 2001. In vitro evaluation of the antimicrobial activity of a castor oil-based irrigant. *J Endod* 27(12): 717-719. doi: 10.1097/00004770-200112000-00001
- Lin, W. H., C. F. Hwang, L. W. Chen and H. Y. Tsen 2006. Viable counts, characteristic evaluation for commercial lactic acid bacteria products. *Food Microbiol* 23(1): 74-81. doi: 10.1016/j.fm.2005.01.013
- Madureira, A. R., M. E. Pintado, A. M. Gomes and F. X. Malcata 2011. Incorporation of probiotic bacteria in whey cheese: decreasing the risk of microbial contamination. *Journal of Food Protection®* 74(7): 1194-1199. doi: 10.4315/0362-028X.JFP-10-217.

- Monteagudo-Mera, A., I. Caro, L. Rodríguez-Aparicio, J. Rúa, M. Ferrero and M. García-Armesto 2011. Characterization of certain bacterial strains for potential use as starter or probiotic cultures in dairy products. *Journal of food protection* 74(8): 1379-1386. doi: 10.4315/0362-028X.JFP-10 392
- Nielsen, D., G. Cho, A. Hanak, M. Huch, C. Franz and N. Arneborg 2010. The effect of bacteriocin-producing *Lactobacillus plantarum* strains on the intracellular pH of sessile and planktonic *Listeria monocytogenes* single cells. *International journal of food microbiology* 141: S53-59. doi: 10.1016/j.ijfoodmicro.2010.03.040
- Nissen-Meyer, J., C. Oppedgård, P. Rogne, H. S. Haugen and P. E. Kristiansen 2010. Structure and mode-of-action of the two-peptide (class-IIb) bacteriocins. *Probiotics and Antimicrobial Proteins* 2(1): 52-60. doi: 10.1007/s12602-009-9021-z
- Onoue, Y., R. Shindo, H. Teranishi, I. Furukawa, Y. Hasegawa and T. Maruyama 2002. Evaluation of selective plating media for the detection of heat- or freeze-injured *Staphylococcus aureus*. *Shokuhin Eiseigaku Zasshi* 43(4): 239-242.
- Pereira, C. A., R. L. Romeiro, A. C. Costa, A. K. Machado, J. C. Junqueira and A. O. Jorge 2011. Susceptibility of *Candida albicans*, *Staphylococcus aureus*, and *Streptococcus mutans* biofilms to photodynamic inactivation: an *in vitro* study. *Lasers Med Sci* 26(3): 341-348. doi: 10.1007/s10103-010-0852-3
- Reder-Christ, K. and G. Bendas 2011. Biosensor applications in the field of antibiotic research—A review of recent developments. *Sensors (Basel, Switzerland)* 11(10): 9450-9466. doi: 10.3390/s111009450
- Rodríguez-Alonso, P., C. Fernández-Otero, J. Centeno and J. Garabal 2009. Antibiotic resistance in lactic acid bacteria and Micrococcaceae/Staphylococcaceae isolates from artisanal raw milk cheeses, and potential implications on cheese making. *Journal of food science* 74(6): M284-293. doi: 10.1111/j.1750-3841.2009.01217.x
- Samelis, J., A. Lianou, E. C. Pappa, B. Bogovič-Matijašić, M. Parapouli, A. Kakouri and I. Rogelj 2014. Behavior of *Staphylococcus aureus* in culture broth, in raw and thermized milk, and during processing and storage of traditional Greek Graviera cheese in the presence or absence of *Lactococcus lactis* subsp. *cremoris* M104, a Wild, Novel Nisin A-producing raw milk isolate. *Journal of Food Protection* 77(10): 1703-1714. doi: 10.4315/0362-028X.JFP-14-105
- Settanni, L., A. Di Grigoli, G. Tornambé, V. Bellina, N. Francesca, G. Moschetti and A. Bonanno 2012. Persistence of wild *Streptococcus thermophilus* strains on wooden vat and during the manufacture of a traditional Caciocavallo type cheese. *International Journal of Food Microbiology* 155(1-2): 73-81 doi: 10.1016/j.ijfoodmicro.2012.01.022.
- Todorov, S. D., D. N. Furtado, S. M. Saad and B. D. Gombossy de Melo Franco 2011. Bacteriocin production and resistance to drugs are advantageous features for *Lactobacillus acidophilus* La-14, a potential probiotic strain. *New Microbiol* 34(4): 357-370.
- Tolinački, M., J. Lozo, K. Veljović, M. Kojić, Đ. Fira and L. Topisirović 2012. Examination of antimicrobial potential in natural isolates of *Lactobacillus casei/paracasei* group. *Genetika* 44(3): 661-677. doi: 10.2298/GENSR1203661T.
- Tuchilus, C., I. Goldu and A. Poiata 2007. *In vitro* activity of Ertapenem against staphylococci. *Rev Med Chir Soc Med Nat Iasi* 111(4): 1035-1039.
- Walsh, C. and S. Fanning 2008. Antimicrobial resistance in foodborne pathogens—a cause for concern? *Current Drug Targets* 9(9): 808-815.

