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REGULAR ARTICLE

ANTIBIOTIC RESISTANCE OF LACTOBACILLI STRAINS ISOLATED FROM MILK AND MILK PRODUCTS FROM MIDDLE SLOVAKIA

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ABSTRACT

The aim of this study was to determine the antibiotic susceptibility of lactobacilli strains isolated from milk and milk products from middle Slovakia. The 42 samples of milk and milk products from sheep farming from middle Slovakia were collected. MRS agar for this cultivation of lactobacilli was used. It was done at 37 °C during 36-48 hours in CO_2 box with 10 % CO_2. Identification of lactobacilli strains by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with Biotyper software – MALDI TOF MS Biotyper was done. Four anitibiotics M.I.C. evaluators strips for antibiotic susceptibility testing were used. These antibiotics were used in this experiment: erythromycin (E 256 – 0.015 µg/ml), ampicillin (AMP 256 – 0.015 µg/ml), gentamycin (CN 256 – 0.015 µg/ml) and tetracycline (TE 256 – 0.015 µg/ml). We isolated 42 lactobacilli strains and identified them as *Lactobacillus plantarum* and *Lactobacillus* spp. We isolated 7 strains of *Lactobacillus plantarum* and 35 isolates were *Lactobacillus* spp. From the seven isolates of *Lactobacillus plantarum* 3 isolates were resistant to ampicillin (MIC 16 µg/ml in 2 isolates and MIC 12 µg/ml in one isolate) and one resistant to tetracycline (MIC 16 µg/ml). From 35 isolates of *Lactobacillus* spp. 9 isolates were resistant to erytromycin (MIC 32 µg/ml), 10 isolates resistant to ampicillin (MIC 16 µg/ml in 4 isolates, MIC 12 µg/ml in 5 isolates and MIC 4 µg/ml in one isolate), 5 isolates resistant to tetracycline (MIC 16 µg/ml). Resistance to gentamycin was not detected. Lactobacilli isolated from milk and milk products were resistant

to erytromycin (resistance was 21.42 %), ampicillin (resistance was 30.95 %) and tetracycline (resistance was 14.28 %) and were sensitive in 100 % to gentamycin.

Keywords: *Lactobacillus* spp., antibiotic resistance, MIC, milk and milk products

INTRODUCTION

Lactobacilli are common microorganisms in food and are also highly represented within the intestinal microbiota of humans and of most animals. They belong to the autochthonous bacteria associated with the mammalian gastrointestinal tract, can contaminate and colonize raw foods (e.g. milk and meat), and multiply during fermentation. They can also survive food processing and persist in finished products, constitute a large portion of the natural microflora in many fermented dairy and meat products. Moreover, lactobacilli display probiotic properties, which lead to their addition to human or animal foodstuffs **(Aguirre and Collins, 1993; Gasser, 1994; Gardiner et al., 1998; Adams, 1999; Mannu et al., 2000; Ouwehand et al., 2002)**. Many lactic acid bacteria (LAB) are resistant to antibiotics. These resistance attributes are often intrinsic and nontransmissible **(Curragh and Collins, 1992; Adams and Marteau, 1995; Charteris et al., 1998b; Salminen et al., 1998)**. However, some LAB may carry potentially transmissible plasmid-encoded antibiotic resistance genes, as shown for example in certain *L. fermentum*, *Lactobacillus plantarum* and *L. reuteri* strains **(Ishiwa and Iwata, 1980; Ahn et al., 1992; Tannock et al., 1994; Fons et al., 1997)**. In the last decade, a concern has arisen that microorganisms used for food and feed production could be vehicles for transmission of antibiotic resistance genes **(EFSA, 2008; Teuber et al., 1999)**. Fermented food products have received increasing attention in recent years as potential vehicles of spread of antibiotic resistance determinants, which might be horizontally transferred to opportunistic pathogens within complex microbial communities such as the gut microflora **(Perreten et al., 1997; Franz et al., 1999; Klein et al., 2000)**. The widespread use of antibiotics in medicine and animal husbandry is the most important factor for the emergence, selection, and dissemination of antibiotic resistant bacteria **(Adam, 2002; McDonald et al., 1997; Witte, 1998; Sarmah et al., 2006)**. Antibiotic resistant bacteria and drug resistance genes have become an important environmental contamination issue which is receiving an increased attention **(Kummerer, 2004; Pruden et al., 2006; Sapkota et al., 2007)**. The antibiotic resistance genes can be transferred between bacteria in the environment

through plasmids, integrons and transposons **(Pang et al., 1994; Schwarz and Chaslus-Dancla, 2001; Nordmann and Poirel, 2005; Pruden et al., 2006)**. **Keyser et al. (2008)** noted that in recent year, accumulating problems with resistant bacteria, lead to predictions that we are back in the period before the discovery of antibiotics. Infections caused by resistant strains of microorganisms evoked costly treatment of animals and humans. Such infections prolong the pathological condition and if are not treated with the right antibiotics may increase mortality **(Witte, 2006)**.

The aim of this study was to determine the susceptibility of lactobacilli strains isolated from milk and milk products from middle Slovakia.

MATERIAL AND METHODS

Collection of samples

The 42 samples of milk and milk products (milk and milk products - sheep cheese, bryndza and parenica) were collected from sheep farming from middle Slovakia. From each samples of milk and milk products one lactobacilli strain was isolated. A 42 strains of lactobacilli in this experiment were isolated. Samples of milk and milk products were collected in June 2011 by sterile swabs cotton (Copan Inovation, Italy). Following, samples were transported in medium to laboratory.

Cultivation of lactobacilli strains

Microorganisms samples were directly spread on the surface of agar by sterille swabs cotton. MRS agar (Biolife, Italy) for cultivation of lactobacilli strains were used. Cultivation of *Lactobacillus* spp. was done at 37 °C during 36-48 hours in CO_2 Unitherm 50 incubator (UNI Equip GmbH, Germany) with 10 % CO_2.

For obtaining of pure culture of lactobacilli strain was done by four-ways streak plate method and same condition for recultivation was used.

Identification of lactobacilli strains by MALDI TOF MS Biotyper

Identification of lactobacilli strains were done by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with Biotyper software – MALDI TOF

MS Biotyper (Bruker Daltonik GmbH, Germany). As recommended by the manufacturer, for matrix-assisted laser desorption/ionization time-of-flight bacterial analysis were used cells from a single colony of fresh overnight culture for each isolating to prepare samples according to the microorganisms profiling ethanol–formic acid extraction procedure. Each sample spot was overlaid with 2 µL of matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50 % acetonitrile with 2.5 % trifluoroacetic acid) and again air-dried for 15 min. To identify microorganisms, the raw spectra obtained for each isolate was imported into BioTyper software, version 2.0 (Bruker Daltonik GmbH, Germany), and analysed without any user intervention **(Kmeť and Drugdová, 2012)**.

MIC susceptibility testing

Four anitibiotics M.I.C. evaluators strips (OXOID, England) for antibiotic susceptibility testing were used. These antibiotics were used in this experiment: erythromycin (E 256 – 0.015 µg/ml), ampicillin (AMP 256 – 0.015 µg/ml), gentamycin (CN 256 – 0.015 µg/ml) and tetracycline (TE 256 – 0.015 µg/ml). The antibiotic susceptibilty of lactobacilli strains were tested in LSM agar consisting 90 % Mueller-Hinton broth (Biolife, Italy) and 10 % MRS broth (Biolife, Italy) with 15 g Agar bios special per liter (Biolife, Italy). Pure 48 hours culture of lactobacilli strains were used for testing of antibiotic. A sterile cotton swabs were dipped in the suspension (1.0 McFarland density) and by swabbing in three directions were used to inoculate an agar plate of the recommended medium. After drying the surface, the MIC strips were placed on the agar plate and incubated micro-aerobically with 10 % CO_2. Determined results were evaluated by **EFSA (2008).**

RESULTS AND DISCUSSION

In this experiment was determined testing of an antibiotic susceptibility of lactobacilli strains isolated from milk and milk products from middle Slovakia. We isolated 42 lactobacilli strains and identified them as *Lactobacillus plantarum* and *Lactobacillus* spp. We isolated 7 strains of *Lactobacillus plantarum* and 35 isolates were *Lactobacillus* spp. From the seven isolates of *Lactobacillus plantarum* 3 isolates were resistant to ampicillin (MIC 16 µg/ml in 2 isolates and MIC 12 µg/ml in one isolates) and one to tetracycline (MIC 16 µg/ml). From 35 isolates of *Lactobacillus* spp. 9 isolates were resistant to erytromycin (MIC 32 µg/ml), 10 to ampicillin (MIC 16 µg/ml in 4 isolates, MIC 12 µg/ml in 5 isolates and MIC 4

µg/ml in one isolate), 5 to tetracycline (MIC 16 µg/ml) and resistance to gentamycin was not detected. Most lactobacilli strains isolated from milk and milk products were sensitive to all antibiotics which we used in this experiment. Susceptibility of wild type of *Lactobacillus* spp. strains to erytromycin ranging between 0.015 and 32 µg/ml, to ampicillin between 0.09 nad 16 µg/ml, to gentamycin between 0.06 and 8 µg/ml and to tetracycline between 0.015 and 16 µg/ml. Also **Bujňáková and Kmeť (2012)** tested *Lactobacillus* strains to gentamycin and erytromycin isolated from dairy products and they determined that MIC values in erytromycin ranging from 0.25 to 0.5 µg/ml and MIC values in gentamycin ranging from 2 to 4 µg/ml. Alike **Mayrhofer et al. (2010)** examined antibiotic resistance in *Lactobacillus acidophilus* group to many antibiotics and they determined that MIC values for ampicillin ranging from 0.12 to 16 µg/ml, for erytromycin from 0.12 to 32 µg/ml, for gentamycin from 0.5 to 8 µg/ml and for tetracycline from 1 to 256 µg/ml. Susceptibility of wild type of *Lactobacillus plantarum* to erytromycin ranging between 0.25 and 0.75 µg/ml, to ampicillin between 0.09 and 16 µg/ml, to gentamycin between 0.25 and 8 µg/ml and to tetracycline 0.015 and 16 µg/ml. The authors **Zhou et al. (2010)** found genatmycin resistance in *Lactobacillus plantarum* obtained from commercial sphere. In our experiment, we don't found resistant strains of lactobacilli to gentamycin. Similarly **Rao Thumu et al. (2012)** found resistant *Lactobacillus plantarum* to erytromycin isolated from milk products. They described that MIC range was to 32 µg/ml. In our experiment was determined MIC range between 0.25 and 0.75 µg/ml in *Lactobacillus plantarum* samples. Also these authors determined tetracycline resistance in *Lactobacillus plantarum* and they decribed that MIC range was to 128 µg/ml, while in our experiment we determined only MIC ranging for tetracycline between 0.015 and 16 µg/ml. More detailed results are described into the Table 1.

Table 1 Antibiotic resistance profile of lactobacilli isolated from milk and milk products

Strains	Antibiotics M.I.C strips (µg/ml)							
	Erytromycin (E 265-0.015)		Ampicillin (AMP 256-0.015)		Gentamycin (CN 256-0.015)		Tetracycline (TE 256-0.015)	
Lactobacillus plantarum	0.375	S	2	S	2	S	4	S
Lactobacillus plantarum	0.375	S	2	S	0.45	S	**16**	**R**
Lactobacillus plantarum	0.25	S	1	S	8	S	0.015	S
Lactobacillus plantarum	0.25	S	**16**	**R**	0.25	S	4	S
Lactobacillus plantarum	0.75	S	0.09	S	0.5	S	1	S
Lactobacillus plantarum	0.25	S	**16**	**R**	8	S	0.5	S
Lactobacillus plantarum	0.75	S	**12**	**R**	0.5	S	6	S
Lactobacillus spp.	**32**	**R**	**12**	**R**	0.375	S	0.25	S

Lactobacillus spp.	0.015	S	0.12	S	0.06	S	**16**	**R**
Lactobacillus spp.	0.015	S	0.09	S	0.06	S	0.5	S
Lactobacillus spp.	**32**	**R**	2	S	2	S	4	S
Lactobacillus spp.	**32**	**R**	2	S	2	S	**16**	**R**
Lactobacillus spp.	0.015	S	0.9	S	0.06	S	1	S
Lactobacillus spp.	**32**	**R**	**4**	**R**	2	S	6	S
Lactobacillus spp.	**32**	**R**	**12**	**R**	0.25	S	0.015	S
Lactobacillus spp.	**32**	**R**	**12**	**R**	8	S	0.015	S
Lactobacillus spp.	**32**	**R**	0.12	S	0.375	S	0.015	S
Lactobacillus spp.	0.015	S	0.09	S	0.06	S	2	S
Lactobacillus spp.	0.015	S	1	S	0.06	S	1	S
Lactobacillus spp.	**32**	**R**	0.9	S	0.25	S	0.5	S
Lactobacillus spp.	0.015	S	0.12	S	0.06	S	1	S
Lactobacillus spp.	0.015	S	0.12	S	0.25	S	**16**	**R**
Lactobacillus spp.	0.06	S	0.09	S	0.25	S	4	S
Lactobacillus spp.	0.05	S	1	S	0.06	S	**16**	**R**
Lactobacillus spp.	0.12	S	1	S	0.5	S	6	S
Lactobacillus spp.	0.25	S	**16**	**R**	0.25	S	0.25	S
Lactobacillus spp.	0.06	S	0.9	S	8	S	**16**	**R**
Lactobacillus spp.	**32**	**R**	1	S	8	S	0.5	S
Lactobacillus spp.	0.015	S	**16**	**R**	0.45	S	0.015	S
Lactobacillus spp.	0.015	S	0.9	S	8	S	4	S
Lactobacillus spp.	0.015	S	2	S	0.375	S	0.12	S
Lactobacillus spp.	0.015	S	**12**	**R**	0.06	S	1	S
Lactobacillus spp.	0.015	S	**12**	**R**	0.06	S	0.015	S
Lactobacillus spp.	0.12	S	0.12	S	8	S	0.015	S
Lactobacillus spp.	0.015	S	0.09	S	0.25	S	0.015	S
Lactobacillus spp.	0.015	S	1	S	0.06	S	4	S
Lactobacillus spp.	0.015	S	**16**	**R**	0.5	S	1	S
Lactobacillus spp.	0.06	S	1	S	0.25	S	0.5	S
Lactobacillus spp.	0.015	S	1	S	2	S	0.5	S
Lactobacillus spp.	0.015	S	**16**	**R**	0.5	S	1	S
Lactobacillus spp.	0.015	S	0.9	S	0.25	S	0.015	S
Lactobacillus spp.	0.015	S	1	S	2	S	4	S

Legend: R – resistant, S - sensitive

Lactobacilli isolated from milk and milk products were resistant to erytromycin (resistance was 21.42 %), ampicillin (resistance was 30.95 %) and tetracycline (resistance was 14.28 %) and to gentamycin lactobacilli strains were sensitive in 100 %. Geometric averages of MIC values for erytromycin was 0.17 μg/ml, ampicillin 1.37 μg/ml, gentamycin 0.49 μg/ml and tetracycline 0.71 μg/ml. More detailed results are described into the Table 2.

The number of researcher, such as **Lira et al. (2004)**, **Picozzi et al. (2005)**, **Caro et al. (2007)** and **Čížek et al. (2007)**, which examined antibiotic resistance of bacteria isolated from different food samples have argued that the results of antibiotic resistance vary from study to study.

Table 2 Antibiotic resistance (R) and minimum inhibitory concentrations (MIC_{50}, MIC_{90}, in micrograms per mililiter) in lactobacilli strains

Antibiotics	R (%)	MICxG*	MIC_{50}	MIC_{90}
Erythromyicin (E)	21.42	0.17	0.06	32
Ampicillin (AMP)	30.95	1.37	1	16
Gentamycin (CN)	0	0.49	0.375	8
Tetracycline (TE)	14.28	0.71	1	16

Legend: *MICxG (geometrical average), E – erytromycin, AMP – ampicillin, CN – gentamycin, TE - tetracycline

CONCLUSION

In conclusion, we determined that lactobacilli strains isolated from milk and milk products were resistant to erythromycin, ampicillin and tetracycline. Also we determined that lactobacilli strains were not resistant to gentamycin. From the results is shown, that antibiotic resistance spreads in environment very well and this resistant non-pathogenic bacteria can be transfer from animal to food and from food to human. Also as shown in the obtained conclusions of many researchers, results of antibiotic resistance vary from study to study.

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