ANTIBACTERIAL SUSCEPTIBILITY OF LACTOBACILLI ISOLATED FROM PORK MEAT OF CONVENTIONAL BREEDING

B. Bobček[1], Ľ. Gajdošová[1], M. Kačániová[2], J. Lejková[1], L. Hleba[1], V. Kmeť[3]

[1]Department of Animal Husbandry, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2 , 949 76 Nitra, Slovakia
[2]Department of Microbiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia
[3]Institute of Animal Physiology, Slovak Academy of Sciences, Soltesovej 4, 040 01 Košice, Slovakia
Corresponding author: bobcek.branislav@uniag.sk
Original scientific paper

Abstract: The aim of this study was monitoring of antibiotic resistance of *Enterobacteriaceae* genera isolated from *musculus longissimus at thoracis* and *musculus semimembranosus* of pork. The pigs was feeding in two groups (control and experimental wit selenium application). For the antibiotic susceptibility testing disk diffusion method was used. *Lactobacillus* species were tested against two antibiotics: ampiciline, tetracycline. For the detection and identification of lactobacilli we used MRS and Rogose agar. For identification of lactobacilli MALDI-TOF-MS were used. The higher resistance of isolated bacteria were found on ampicillin (29%) and on tetracycline (46%) in experimental group. The lower resistance of isolated bacteria were found on ampicillin (25%) and on tetracycline (35%) in control group. The most resistance bacteria of *Lactobacillus* genus was *Lactobacillus plantarum* in control and experimental group, too. From MLT and MSM pork meat *Lactobacillus* species *Lactobacillus reuteri, L. crispatus, L.paracasei, L. farcimins* and *L curvatus* and from non-lactobacilli *Lactococcus lastis, Pediococcus pentosaceus* were isolated (Table 2). The higher isolated species from lactobacilli genera was *Lactobacillus paracasei* in control group with 35.63 % and *Lactobacillus reuteri* in experimental group with 25.83%.

Key words: antibiotics, resistance, lactobacilli, pork meat

Introduction

Animal muscles turn into meat by a number of chemical and structural processes. By definition, meat conditioning starts at the moment of animal death and ends with the exhaustion of degradable energy-rich compounds such as ATP,

creatine and glycogen. Biochemical changes undergoing during refrigerated conditioning cause muscle structural alterations which are of paramount importance for meat quality. Immediately after death, skeletal muscle is soft but it soon becomes very tough and unpalatable from rigor mortis. As a result of animal death the supply of energy-rich compounds and oxygen stops. However, these compounds continue being anaerobically degraded in the muscle cell conducting to a pH fall between 5.3 and 5.8 and the onset of rigor mortis in the meat. These changes produce a shortening in muscles fibers with an adverse effect on meat tenderness *(Honikel, 1992)*. Muscle toughness gradually decreases due to protein degradation, and meat texture is improved during postmortem conditioning *(Nishimura, 1998, 2002)*.

Resistance to antimicrobial substances has become a serious clinical problem, prompted by the extensive use of antimicrobial substances for human and veterinary medicine as well as in animal growth promoters. Lactic acid bacteria (LAB) naturally occur in foods and play a key role in the manufacture of fermented foods as well as in food preservation. They are also commensal bacteria in humans and animal microflora *(Salminen et al., 1993)*, and many isolates find specific applications as probiotics *(Isolauri et al., 2004)*. Although lactobacilli are seldom associated with infections *(Fruchart et al., 1997; Adams, 1999)*, there is a growing concern that they may serve as reservoirs for antimicrobial resistance genes, and the presence of resistance genes as well as the transfer of conjugative plasmids and transposons to and from lactobacilli have been reported *(Teuber et al., 1999)*.

The objective of our study was to determine the antibiotic resistance of lactobacilli isolated from *musculus longissimus at thoracis* and *musculus semimembranosus* of pork, as well as determine species spectrum of lactobacilli from meat of pigs.

Materials and Methods

Antibiotic resistance study was done of lactobacilli isolated from *musculus longissimus at thoracis* MLT and *musculus semimembranosus* MSM of pigs from conventional breeding from Slovakia. From pigs breeding farm were obtained 30 animals (15 animals from control group and 15 animal from group with selenium application).

In our study, we identified strains which were resistant and susceptible to antibiotics. Bacterial strains were isolated from meat and collected with a kit containing the swab (Copan Inovation, Brescia) and transported in medium to laboratory Department of Microbiology, Faculty of Biotechnology and Food Science in Slovak University of Agriculture in Nitra. Samples were suspended in physiological solution.

For cultivation of lactobacilli MRS and Rogose agar (Biomark, Pune) was used. Incubation was conducted for 48 hours at 37°C. We repeat this step until we have completely cleaned culture of strains from *Lactobacillus* genera.

The pure inoculum of strains of *Lactobacillus* genera was prepared by suspending of colonies from the agar plates and suspension was adjusted to equal a 2 McFarland standard. The sensitivity of all strains of *Lactobacillus* genera was tested against: ampicilline AMP and tetracycline TE. The incubation of strains was done at the temperature 37 °C.

For the antibiotic suspcebility M.I.C.E were used (Oxoid, UK). M.I.C.EvaluatorTM (M.I.C.E.) is a system for quantitatively determining the Minimum Inhibitory Concentration (MIC) of an antibiotic against a test organism. The Oxoid M.I.C.E. provides a gradient of antibiotic stabilized on a plastic strip with 30 graduations, to give an accurate MIC over the range 256 μg ml^{-1} – 0.015 μg ml^{-1}. For inhibition zones M.I.C.Evaluator Interpretation Guide were used.

For lactobacilli identification MALDI-TOF-MS were used. Fast and reliable identification of microorganisms is a crucial step toward an appropriate treatment of infectious diseases in medical and veterinary diagnostics and is of main interest today. Mass spectral identification of microorganisms has been shown as a tool for rapid identification. The most widely applied technology for mass spectral identification of microorganisms is matrix assisted laser desorption ionization time-of-flight mass spektrometry (MALDI-TOF-MS). Identification of microorganisms by MALDI-TOF-MS can be applied to a wide array of bacteria, yeasts, and moulds. A small amount of cells from a plate is sufficient for an automated and rapid identification.

Results and Discussion

European consumers generally have positive attitudes to pork production that may or may not *(Ngapo et al., 2003)* be linked to higher consumption of pork. Pork is seen as suitable for different dishes although not a meat for special occasions *(Bryhni et al., 2002)* and may be perceived by some as being relatively fatty and unhealthy compared to beef and poultry *(Verbeke et al., 1999)*.

Lactobacilli are ubiquitous lactic acid bacterium commonly associated with the food environment. Although this organism was isolated from various fermented vegetables such as sauerkraut and kimchi *(Choi et al., 2003; Fadda et al., 2010)* and fish products *(Najjari et al., 2008)*, it was mostly isolated from the meat environment *(Chenoll et al., 2007; Fontana et al., 2005, 2006)*.

In our experiment in control group 14 species of bacteria and in experimental group 14 species were isolated. Percentages of *Lactobacillus* species of pork meat showed Table 1. The higher resistance of isolated bacteria were found on ampicillin (29%) and on tetracycline (46%) in experimental group. The lower

resistance of isolated bacteria were found on ampicillin (25%) and on tetracycline (35%) in control group. The higher sensitivity of isolated bacteria were found on ampicillin in control group (65%) and in experimental group (59%). The sensitivity of isolated bacteria were found on tetracycline in control group (45%) and in experimental group (46%), too. The most resistance bacteria of *Lactobacillus* genus was *Lactobacillus plantarum* in control and experimental group, too.

Escherichia coli is the main representative of the *Enterobacteriaceae* genera. The high values of resistance to ampicillin (100 %) of all tested strains of *E. coli* O 157.

Table 1 Percentages of lactobacilli isolated from pork meat susceptible (S), intermediate (I) and resistant (R) to antimicrobial agents by disk diffusion methods

Antimicrobial agent	Control group			Experimental group		
(μg)	S	I	R	S	I	R
ampicillin	65%	10%	25%	59%	12%	29%
tetracycline	45%	20%	35%	46%	8%	46%

From MLT and MSM pork meat *Lactobacillus* species *Lactobacillus reuteri, L. crispatus, L.paracasei, L. farcimins* and *L curvatus* and from non-lactobacilli *Lactococcus lastis, Pediococcus pentosaceus* were isolated (Table 2). The higher isolated species from lactobacilli genera was *Lactobacillus paracasei* in control group with 35.63 % and *Lactobacillus reuteri* in experimental group with 25.83 %.

Table 2 Genera distribution of *Lactobacillus* genera and non-lactobacilli isolated from pork meat

Microorganisms	Control group %	Experimental group%
Lactobacillus reuteri	22.23	25.83
Lactobacillus crispatus	20.25	21.25
Lactobacillus paracasei	35.63	21.63
Lactobacillus curvatus	7.86	9.63
Lactobacillus farciminis	6.21	7.13
Lactococus lactis	5.26	7.62
Pediococcus pentosaceus	2.56	6.91

In the study *Fadda et al. (2008)* were spread LAB cells onto meat discs surfaces at an initial value ranging from 4 to $7x10^8$ $CFU.cm^{-2}$ and increased between 1 and 2 log after 15 days of storage at 7 °C under vacuum-packaged

conditions. Although *L. curvatus* CRL705 cell counts were 1-log higher than *L. sakei* 23K (3.0x10^{10} vs. 3.5x10^9 CFU.cm^{-2}).

The percentage of the *Lactobacillus* isolates from caper fermentations that were able to grow in liquid medium at given antimicrobial concentrations. All lactobacilli isolates were sensitive to ampicillin (MIC > 8 μg ml^{-1}), but a high percentage of *L. plantarum* isolates were resistant to penicillin (MIC > 8 μg ml^{-1}). These results are in agreement with those reported by *Zarazaga et al. (1999)*, although generally lactobacilli seem to be sensitive to penicillins according to Danielsen and Wind (2003). All isolates were resistant to vancomycin and teicoplanin (MIC >16 μg ml^{-1}). Many species of *Lactobacillus* carry intrinsic resistance towards vancomycin which is due to the presence of D-alanine: D-alanine ligase-related enzymes *(Elisha and Courvalin, 1995)*. Intrinsic vancomycin resistance is not considered a risk factor in lactobacilli, as many strains have a long history of safe use as probiotics and there is no indication that they could transfer resistance to other species *(Mattila- Sandholm et al., 1999)*. Rifampicin resistance was also detected in many isolates of *L. plantarum* but not in *L. brevis* or *L.fermentum*, indicating species-associated resistance. High-level resistance to ciprofloxacin (MIC > 2 μg ml^{-1}) was detected in all isolates and low level resistance to levofloxacin (MIC >2 μg ml^{-1}) was also detected in most *L. plantarum* and all *L. brevis* isolates, but not in *L. fermentum*. Previous reports also indicate a poor activity of ciprofloxacin and levofloxacin against many *Lactobacillus* isolates *(Hamilton-Miller and Shah, 1994; Herra et al., 1995; Zarazaga et al., 1999)*. Several isolates of *L. plantarum* and most *L. brevis* strains were resistant to low concentrations of nitrofurantoin (MIC > 32 μg ml^{-1}). However, all isolates were inhibited by gentamicin (MIC > 500 μg ml^{-1}), streptomycin (MIC > 1000 μg ml^{-1}), and quinupristin/dalfopristin (MIC > 1 μg ml^{-1}). The protein synthesis inhibitors erythromycin and chloramphenicol were highly active against all isolates. However, a high percentage of *L. plantarum* and all *L. brevis* isolates showed intermediate resistance to tetracycline (MIC > 4 μg ml^{-1}) and a low percentage were resistant (MIC >8 μg ml^{-1}). Tetracycline resistance was corroborated with E-test *(Pulido et al., 2005)*. The plasmid location of tetracycline resistance determinants in *L. plantarum* has been reported *(Danielsen, 2002)* and transfer of tetracycline resistance from *Lactobacillus* to other Gram-positive bacteria has been demonstrated *in vitro (Gevers et al., 2003)*. Although this study did not investigate the source of tetracycline resistence in lactobacilli isolated from fermented capers, previous studies have shown that a tetracycline resistance region, containing a tet(M) gene in *L. plantarum* plasmid pMD5057, had high homology to sequences from *Clostridium perfringens* and *Staphylococcus aureus (Danielsen, 2002)*. This region also contained an IS-element. Bacteria containing similar plasmids or IS-elements are commonly isolated from vegetables but also from other habitats *(Danielsen, 2002)*. Since tetracycline is heavily used in human and animal medicine, in aquaculture, and even as a growth promoter or sprayed onto fruit trees

in some countries *(Chopra and Robert, 2001)*, it is possible that tetracycline resistance determinants reach lactobacilli found on plant material from different sources. According to results from this study, the capacity of lactobacilli isolated from caper fermentations to spread tetracycline resistence to other bacteria with higher pathogenic potentials should be investigated in further detail, and the selection of strains free of resistance determinants should be recommended in the development of starter cultures to be used in caper fermentation.

Conclusion

In this study in MLT and MSN pork meat *Lactobacillus* species *Lactobacillus reuteri, L. crispatus, L.paracasei, L. farcimins* and *L curvatus* and from non-lactobacilli *Lactococcus lastis, Pediococcus pentosaceus* were isolated. The higher isolated species from lactobacilli genera was *Lactobacillus paracasei* in control group with 35.63 % and *Lactobacillus reuteri* in experimental group with 25.83 %. The antimicrobial resistance showed diverse patterns depending on the bacteria. This study provides scientific database to control antimicrobial resistant bacteria in pork meat processing chain from slaughterhouses to retail shops.

Acknowledgment

Antibakterijska osetljivost bakterija mlečne kiseline izolovanih u mesu svinja odgajanih na konvencionalan način

B. Bobček, Ľ. Gajdošová, M. Kačániová, J. Lejková, L. Hleba, V. Kmeť

Rezime

Cilj ove studije bio je praćenje antibiotske rezistencije *Enterobacteriaceae* rodova izolovanih iz *musculus longissimus at thoracis* i *musculus semimembranosus* iz svinjskog mesa. Svinje su hranjene u dve grupe (kontrolna i ogledna sa dodatkom selena). Za ocenu antibiotske osetljivosti korišćeno je testiranje disk-difuzionom metodom. *Lactobacillus* vrste testirane su u odnosu na dva antibiotika: ampiciline, tetracycline. Za detekciju i identifikaciju laktobacila koristili smo MRS i Rogose agar. Za identifikaciju laktobacila korišćen je MALDI-TOF-MS. Veća rezistencija izolovanih bakterija utvrđena je na ampicillin (29%) i na tetracycline (46%) u eksperimentalnoj grupi. Niža rezistencija izolovanih bakterija utvrđena je naampicillin (25%) i na tetracycline (35%) u

kontrolnoj grupi. Najrezistentnija bakterija iz roda *Lactobacillus* bila je *Lactobacillus plantarum* u kontrolnoj i eksperimentalnoj grupi, takođe. Iz MLT i MSM svinjskog mesa izolovane su *Lactobacillus vrste- Lactobacillus reuteri, L. crispatus, L.paracasei, L. farcimins i L curvatus* a od ne-laktobacila *Lactococcus lastis, Pediococcus pentosaceus* (tabela 2). Najčešće izolovane vrste iz roda *Lactobacillus* bile su *Lactobacillus paracasei* u kontrolnoj grupi sa 35,63 % i *Lactobacillus reuteri u* eksperimentalnoj grupi sa 25,83%.

References

ADAMS M.R. (1999): Safety of industrial lactic acid bacteria. Journal of Biotechnology, 68, 171-178.

BRYHNI E.A., BYRNE D.V., RØDBOTTEN M., CLAUDI-MAGNUSSEN C., AGERHEM H., JOHANSSON M., LEA P., MARTENS M. (2002): Consumer perceptions of pork in Denmark, Norway and Sweden. Food Quality and Preference, 13, 257-266.

DANIELSEN M. (2002): Characterization of the tetracycline resistence plasmid pMD5057 from *Lactobacillus plantarum* 5057 reveals a composite structure. Plasmid, 48, 98-103.

DANIELSEN M., WIND A. (2003). Susceptibility of Lactobacillus spp. to antimicrobial agents. Int. J. Food Microbiol., 82, 1-11.

ELISHA B.G., COURVALIN P. (1995): Analysis of genes encoding D-alanine: D-alanine ligase-related enzymes in Leuconostoc mesenteroides and *Lactobacillus* spp., Gene, 152, 79-83.

FADDA S., CHAMBON C., CHAMPOMIER-VERGE`S M.C., TALON R.G. (2008): Vignolo a Lactobacillus role during conditioning of refrigerated and vacuum-packaged. Argentinean Meat Science, 79, 603-610.

FADDA S., ANGLADE P., BARAIGE F., ZAGOREC M., TALON R., VIGNOLO G., CHAMPOMIER-VERGÈS M. CH. (2010): Adaptive response of *Lactobacillus sakei* 23K during growth in the presence of meat extracts. A proteomic approach International. Journal of Food Microbiology, 142, 36-43.

FONTANA C., COCCONCELLI P., VIGNOLO G. (2005): Monitoring the bacterial population dynamics during fermentation of artisanal Argentinean sausages. International Journal of Food Microbiology, 103, 131-142.

FONTANA C., COCCONCELLI P., VIGNOLO G. (2006): Direct molecular approach to monitoring bacterial colonization on vacuum-packaged beef. Applied and Environmental Microbiology, 72, 5618-5622.

FRUCHART C., SALAH A., GRAY C., MARTIN E., STAMATOULLAS A., BONMARCHAND G., LEMELAND J.F., TILLY H. (1997): Lactobacillus species as emerging pathogens in neutropenic patients. Eur. J. Clin. Microbiol. Infect. Dis., 16, 681-684.

GEVERS D., HUYS G., SWINGS J. (2003): In vitro conjugal transfer of tetracycline resistance from Lactobacillus isolates to other Gram-positive bacteria. FEMS Microbiol. Lett., 225, 125-130.

HAMILTON-MILLER J.M.T., SHAH S. (1994): Susceptibility patterns of vaginal lactobacilli to eleven oral antibiotics. J. Antimicrob. Chemother., 33, 1059-1060.

HERRA C.M., CAFFERKEY M.T., KEANE C.T. (1995): The in-vitro susceptibilities of vaginal lactobacilli to four broad spektrum antibiotics, as determined by the agar dilution and E-test methods. J. Antimicrob. Chemother., 35, 775-783.

HONIKEL K.O. (1992): The biochemical basis of meat conditioning. In SMULDERS J., FRANS M., TOLDRA´ F., FLORES J., PRIETO M. (eds.), New technologies for meat and meat products, 135-161.

CHENOLL E., MACIÁN M., ELIZAQUÍVEL P., AZNAR R. (2007): Lactic acid bacteria associated with vacuum-packed cooked meat product spoilage: population analysis by rDNA-based methods. Journal of Applied Microbiology, 102, 498-508.

CHOI I.K., JUNG S.H., KIM B.J., PARK S.Y., KIM J., HAN H.U. (2003): Novel Leuconostoc citreum starter culture system for the fermentation of kimchi, a fermented cabbage product. Antoine van Leeuwenhoek, 84, 247-253.

CHOPRA I., ROBERT M. (2001): Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev., 65, 232-260.

ISOLAURI E., SALMINEN S., OUWEHAND A.C. (2004): Microbial–gut interactions in health and disease. Probiotics. Best Pract. Res. Clin. Gastroenterol., 18, 299-313.

MATTILA-SANDHOLM T., MÄTTO J., SAARELA M. (1999): Lactic acid bacteria with health claims-interactions and interference with gastrointestinal flora. Int. Dairy J., 9, 25-35.

NAJJARI A., OUZARI H., BOUDABOUS A., ZAGOREC M. (2008): Method for reliable isolation of Lactobacillus sakei strains originating from Tunisian seafood and meat products. International Journal of Food Microbiology, 121, 342-351.

NGAPO T.M., DRANSFIELD E., MARTIN J.F., MAGNUSSON M., BREDAHL L., NUTE G.R. (2003): Consumer perceptions: pork and pig production. Insights from France, England, Sweden and Denmark. Meat Science, 66, 125-134.

NISHIMURA T. (1998): Mechanism involved in the improvement of meat taste during post mortem ageing. Food Science and Technology International, 4, 241-249.

NISHIMURA T. (2002): Influence of the peptides produced during post mortem conditioning on improvement of meat flavor. In: TOLDRA´ F. (ed), Research advances in the quality of meat and meat products. Trivandrum, India: Research Singpost (Chapter 4), 65-78.

PULIDO R.P., OMAR N.B., LUCAS R., ABRIOUEL H., KAŇAMERO M.M., GÁLVEZ A. (2005): Resistance to antimicrobial agents in lactobacilli isolated from caper. Fermentations Antonie van Leeuwenhoek, 88, 277-281.

SALMINEN S., DEIGHTON M., GORBACH S. (1993): Lactic acid bacteria in health and disease. In: SALMINEN S., WRIGHT A.V. (eds), Lactic acid bacteria. Marcel Dekker, Inc., New York, N.Y., 199-225.

TEUBER M., MEILE L., SCHWARZ F. (1999): Acquired antibiotik resistance in lactic acid bacteria from food. Antonie Leeuwenhoek, 76, 115-137.

VERBEKE W., VAN OECKEL M.J., WARNANTS N., VIAENE J., BOUCQUE´ CH.V. (1999): Consumer perceptions, facts and possibilities to improve acceptability of health and sensory characteristics of pork. Meat Science, 53, 77-99.

ZARAZAGA M., SAINZ Y., PORTILLO A., TENORIO C., RUIZ-LARRARA R.F., CAMPO R.DEL., BAQUERO F., TORRES C. (1999): In vitro activities of ketolide HMR3647, macrolides, and other antibiotics against Lactobacillus, Leuconostoc and Pediococcus isolates. Antimicrob. Agents Chemother., 43, 3039-3041.