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COMPARISON OF ANTIBIOTIC RESISTANCE BETWEEN ECOLOGICAL AND CONVENTIONAL BREEDING IN *ENTEROBACTERIACEAE* GENERA ISOLATED FROM MILK AND MILK PRODUCTS

L. Hleba, M. Kačániová, S. Kunová, M. Kluz, M. Mellen

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Abstract

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The aim of this study was to determine and compare antibiotic resistance of *Enterobacteriaceae* genera isolated from milk and milk products like cheese, bryndza, srvátka and parenica from ecological and conventional breeding from different regions of Slovakia. Distance between breedings was about 20 km. In the both breeding were not used antibiotics. The *Enterobacteriaceae* isolates were tested for susceptibility to three antibiotics ampicillin (AMP), nalixid acid (NA 30) and chloramphenicol (C 30). In our study, we determined that the highest resistance of *Enterobacteriaceae* strains was to ampicillin (100 %) in conventional breeding. The higher resistance in conventional breeding is probably due to greater anthropogenic influences. In ecological breeding we determined 84.61% resistance to ampicillin. The highest susceptibility was to chloramphenicol and nalixid acid (100 %) in conventional breeding. In the ecological breeding resistance to chloramphenicol was 15.38 % and resistance to nalixid acid 7.69 % from all tested isolates. Conversely, higher resistance to chloramphenicol and nalixid acid in the ecological breeding may be due to the persistence of resistant genes in environment. The lowest resistance to chloramphenicol and nalixid acid in conventional breeding may be due to the prohibition of the use of antibiotics as growth promoters. From this genera, we identified *Klebsiella oxytoca, Serratia odorifera* bv.1*, Serratia odorifera* bv. 2*, Citrobacter braakii* and *Escherichia coli* from conventional breeding which were resistant to ampicillin and isolated from cheese, bryndza and parenica. In the ecological breeding we identified this strains: *Raoultella ornithinolytica* resistant to ampicillin, *Serratia rubidaea*, which was resistant to all of used antibiotics and this strains were isolated from milk samples and *Klebsiella pneumoniae*, *Escherichia coli*, *Klebsiella oxytoca* which were resistant to ampicillin and were isolated from cheese samples. The results show that the bacteria can transfer resistance genes to others bacteria, for example to pathogens too. Also that resistant bacteria and their resistant genes survive in the environment and transfer to others animals and products thereof.

antibiotic resistance, *Enterobacteriaceae*, milk and milk products, ecological and conventional breeding

Antibiotic resistance is very dangerous healthy, social and economic problem of the world. Antibiotic resistance of bacteria is a biological danger, which increases morbidity and mortality of animals and human (EFSA, 2008). Antibiotic resistance is a bacterial ability to resist inhibiting or lethal effects of antibiotics. Primary resistance is a genetically determined un-susceptibility against antibiotics without prior contact with antibiotic (Key *et al.*, 2000). Endogenous bacterial flora may play an important role as acceptor and donor of transmissible drug resistance genes (Davies *et al.*, 1994; Sunde *et al.*,

1998). *Enterobacteriaceae* genera are commonly found in the intestinal tract of humans and animals (Sunde *et al*., 1998; Tannock *et al*., 1995) and can also be implicated in human and animal infectious diseases (Threlfal *et al*., 1998).

Enterobacteriaceae are widespread in the environment and many mesophilic species contaminate food in low numbers (Molin and Stenström, 1984; Ternström and Molin, 1987; Garcia-Armesto *et al*., 1993). Görner and Valík (2004) contamination of raw milk distributed to primary and secondary. The primary source of bacterial contamination classify udder cisterns and udder channels, microorganisms from the surface of the udder, of the body, from excrement of dairy cow, from feed and dust, as well as hands and clothing milker, microorganisms from surfaces which are in contact with milk milking, traffic filtering, cooling and transport to the dairy factory. Secondary contamination depends on the starting milk cooling, temperature and cooling time. Keyser *et al*. (2008) note that in recent years, accumulating problems with resistant bacteria, leading to predictions that we are back the period before the discovery of antibiotics. One of way around this problem is to introduce new antibacterial preparation which operates on a locking mechanism of virulence. More precisely, a type III (T3SS) secretion system. Infections caused by resistant strains of microorganisms causing costly treatment of animals and humans. Such infections prolong the pathological condition and if not treated with the right antibiotics may be increased mortality (Witte, 2006).

The aim of this study was compare antibiotic resistance between ecological and conventional breeding in *Enterobacteriaceae* genera isolated from milk and milk products from Slovakia and monitoring of active genes of resistance in bacteria present in environment, animals and food.

MATERIAL AND METHODS

Antibiotic resistance study was done on *Enterobacteriaceae* genera isolated from milk and milk products (cheese, bryndza, srvátka and parenica) from ecological and conventional breeding from different regions of Slovakia. Distance between breedings was about 20 km. From the ecological breeding were obtained samples (n = 36) of milk (n = 18), cheese (n = 12), bryndza (n = 6) and from the conventional breeding were obtained samples (n = 30) of cheese (n = 24), bryndza (n = 3) and parenica (n = 3). This experiment and every sample was done in triplicate repetition and was done the average of resulted values. In our study, we identified only strains resistant to antibiotics. Between ecological and conventional breeding was done to compare. Bacterial strains were isolated from milk and milk products 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 *Enterobacteriaceae* genera MacConkey agar (Biomark, Pune) was used. The pure inoculum of strains of *Enterobacteriaeceae* genera was prepared by suspending of colonies from the agar plates and suspension was adjusted to equal a 0.5 McFarland standard. The sensitivity of all strains of *Enterobacteriaceae* genera was tested against: ampicillin (AMP 10) 10 μg.disc^{-1}, chloramphenicol (C 30) 30 μg.disc^{-1} and nalixid acid (NA 30) 30 μg.disc^{-1}. We used disc diffusion methods according (EUCAST – European committee on antimicrobial susceptibility testing). The incubation of strains was done at the temperature 37 °C. The interpretation of inhibition zones around the disc was according to EUCAST. The inhibition zones were controlled with the reference *Escherichia coli* ATCC 25922. Initial identification of strains of *Enterobacteriaceae* genera were done on the Triple sugar iron agar iso (Biolife, Italiana). Biochemical identification of strains of *Enterobacteriaceae* genera was done by ENTEROtest 24 (Pliva, Lachema). Evaluation of biochemical tests was done by identifying computer program TNW Lite 7.0 software (Pliva, Lachema). From the obtained data we calculated using statistical program STATGRAPHICS basic variation-statistical values like average, standard deviation, minimum, maximum, coefficient of variation and frequency of the size of inhibition zones.

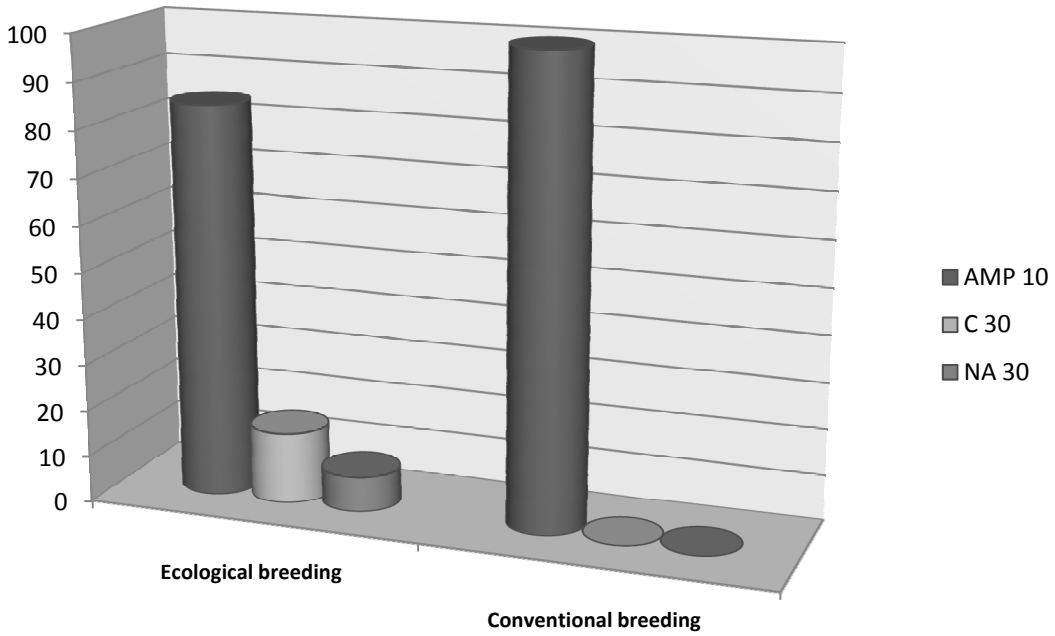
RESULTS AND DISCUSSION

We studied antibiotic resistance in commensal strains of *Enterobacteriaceae* genera, which are considered a potential reservoirs for resistant genes in farm animal. On-farm reservoirs of resistant bacteria provide a potential sources for resistant genes transfer between bacteria as well as an environment for dissemination to new animals, environments and food products. Therefore, identifying these reservoirs and mechanisms of persistance will be a key to reducing the load of resistant bacteria in everywhere.

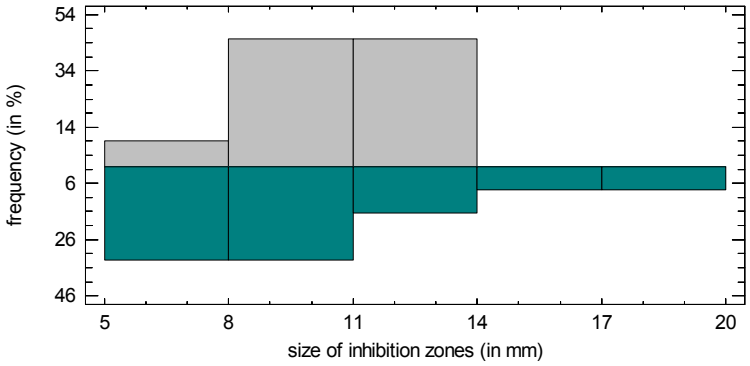
In our study we studied antibiotic resistance of *Enterobacteriaceae* genera strains isolated from milk and milk products from ecological and conventional breeding. We find differences of antibiotic resistance between ecological and conventional breeding. In this study we determined, that in conventional and ecological breeding antibiotic resistance was in high percent to ampicillin (AMP 10). In ecological breeding resistance of bacteria to ampicillin was 84.61%. The high values of resistance to ampicillin (100%) of all tested strains of *E. coli* O 157 Solomakos *et al*. (2009) reported. In conventional breeding resistance to ampicillin was 100%, in all isolates. Farzana *et al*. (2009) reported the similar results, but samples were isolated from Indian milk and products thereof. They determined 100% resistance to ampicillin in *Escherichia coli* mainly. Other microorganisms were less resistant to ampicillin. The lowest resistance 0% to chloramphenicol (C 30) and nalixid acid (NA 30) was in conventional breed-

ing. Also Farzana *et al.* (2009) determine high values of resistance to chloramphenicol in more bacterial strains like *Klebsiella* (more than 60 %), *Enterobacter* (more than 50 %), but mainly in *E. coli* (100 %). In ecological breeding resistance to chloramphenicol was 15.38% and resistance to nalixid acid 7.69% from all tested isolates. The higher values of resistance to chloramphenicol (44.82%) determined Solomakos *et al.* (2009), because 13 of 29 strains (LFH1 – LFH29) of *E. coli* O 157 were resistant to chloramphenicol. The similar results determined Dupont *et al.* (1978), which they tested antibiotic resistance to chloramphenicol in *E. coli*. The values of resistance are shown in the figure No. 1.

Conversely, the highest susceptibility was in conventional breeding. The strains of bacteria, which we find present in products of the conventional breeding were susceptible to chloramphenicol and nalixid acid in 100%. Susceptibility to ampicillin was not determined in samples from conventional breeding. In the ecological breeding was a susceptibility to antibiotics in the high values too. Sus-

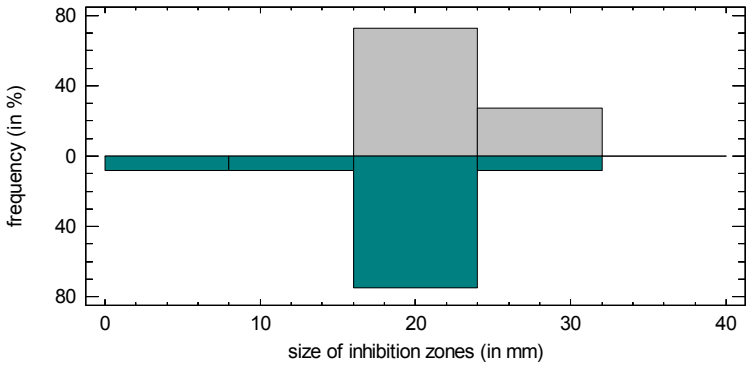


1: *Compare antibiotic resistance between ecological and conventional breeding (in %)*



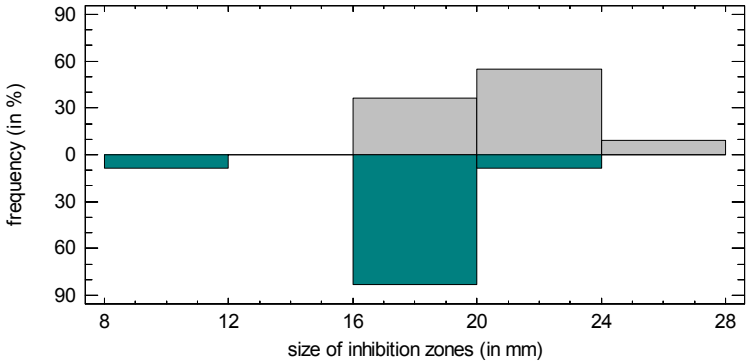
Legend: – ampicillin in ecological breeding, – ampicillin in conventional breeding

2: *Frequency of the size of inhibition zones at the ampicillin*



Legend: – chloramphenicol in ecological breeding, – chloramphenicol in conventional breeding

3: *Frequency of the size of inhibition zones at the chloramphenicol*



Legend: – nalixid acid in ecological breeding, – nalixid acid in conventional breeding

4: *Frequency of the size of inhibition zones at the nalixid acid*

ceptibility to chloramphenicol was 84.62% and susceptibility to nalixid acid 92.31%. Susceptibility of bacteria to ampicillin in ecological breeding was 15.39% only. The several researches like Lira *et al*. (2004), Picozzi *et al*. (2005), Caro *et al*. (2007) and Čížek *et al*. (2007), who researched antibiotic resistance in *E. coli*, respectively in *Enterobacteriaceae* genera isolated from milk and milk products have argued, that results of antibiotic resistance vary from study to study. The values of the susceptibility are shown in the figure No. 1 too.

In the conventional breeding we determined these strains: *Klebsiella oxytoca* (n = 6), *Serratia odorifera* bv. 1 (n = 6), *Serratia odorifera* bv. 2 (n = 6) and *Citrobacter braakii* (n = 3), *Escherichia coli* (n = 9). All of this microorganisms were resistant to ampicillin and susceptible to chloramphenicol and nalixid acid. All of this strains were isolated from cheese samples, except *Escherichia coli*, which was isolated from bryndza and parenica. We not identified strains, which were no resistant to antibiotics. Results are shown in the table No. I.

In the ecological breeding we determined these strains: *Raoultella ornithinolytica* (n = 9) resistant to ampicillin, *Serratia rubidaea* (n = 9) multiresistant to all of used antibiotic, which were isolated from milk samples and *Klebsiella oxytoca* (n = 4) resistant to ampicillin, *Klebsiella pneumoniae* (n = 4) resistant to ampicillin and *Escherichia coli* (n = 4) resistant to ampicillin isolated from cheese samples. We not identified strains, which were not resistant to antibiotics. Results are shown in the table No. II.

Statistical evaluation of inhibition zones, we determined that the greatest variability was in the sam-

I: *Identified strains of Enterobacteriaceae genera isolated from milk and milk products from conventional farming*

Strains	Resistant to	Percentage of identification by TNW software	Isolated from	Number of isolates
Klebsiella oxytoca	ampicillin	98.96%	Cheese	6
Serratia odorifera bv. 1	ampicillin	96.92%	Cheese	6
Serratia odorifers bv. 2	ampicillin	100%	Cheese	6
Citrobacter braakii	ampicillin	97.42%	Cheese	3
Escherichia coli	ampicillin	100%	Bryndza Parenica	9

II: *Identifying strains of Enterobacteriaceae genera isolated from milk and milk products from ecological farming*

Strains	Resistant to	Percentage of identification by TNW software	Isolated from	Number of isolates
Raoultella ornithinolytica	ampicillin	99.72%	Milk	9
Serratia rubidaea	ampicillin nalixid acid chloramphenicol	100%	Milk	9
Klebsiella oxytoca	ampicillin	96.97%	Cheese	4
Klebsiella pneumoniae	ampicillin	98.80%	Cheese	4
Escherichia coli	ampicillin	99.98%	Cheese	4

III: *The basic variation-statistical values of inhibition zones*

Variation-statistical values / antibiotics	AMPeko	AMPcon	C30eko	C30con	NA30eko	NA30con
Average	10.25	11.18	18.92	22.90	18.33	21.45
Standard deviation	4.22	2.23	5.05	3.86	3.11	2.70
Coefficient of variation	41.21%	19.92%	26.71%	16.84%	16.99%	12.57%
Minimum	6	6	6	20	9	18
Maximum	19	14	25	31	22	27

ples from ecological breeding, when we used ampicillin (41.21%). Minimum and maximum values of inhibition zones ranged from 6 to 19 mm and average of inhibition zone was 10.25 mm. The lowest variability was in the samples from conventional breeding, when we used nalixid acid (12.57%). Minimum and maximum values of inhibition zones ranged from 18 to 27 mm and average of inhibition zone was 21.45 mm. Others variation-statistical values are shown in the table No. III.

The highest frequency of the size of inhibition zones in the conventional breeding at ampicillin was in range of 5 to 11 mm and in ecological breeding at ampicillin in range of 8 to 14 mm (figure No. 2). At the chloramphenicol in the conventional and ecological breeding was the highest frequency of the size of inhibition zones in range of 16 to 24 mm (figure No. 3). At the nalixid acid in the conventional breeding was the highest frequency of the size of inhibition zones in range of 20 to 24 mm and in the ecological breeding in range of 16 to 20 mm (figure No. 4).

SUMMARY

The aim of this study was compare antibiotic resistance in *Enterobacteriaceae* genera isolated from milk and milk products from ecological and conventional breeding and monitoring of active genes of resistance in bacteria present in environment, animals and food. Distance between breedings was about 20 km. We used plates method for cultivation of *Enterobacteriaceae* on the MacConkey agar. For the susceptibility testing we used disc diffusion method and interpretation of inhibition zones around the disc by EUCAST Clinical Breakpoint Table v. 1.1 2010-04-27. For initial identification we used Triple sugar iron agar iso and for detail identification biochemical tests ENTEROtest 24. We determined 100% resistance to ampicillin in *Enterobacteriaceae* genera from conventional breeding and 84.61% resistance to ampicillin from ecological breeding. In ecological breeding resistance to chloramphenicol was 15.38% and resistance to nalixid acid 7.69% from all tested isolates. In conventional breeding was resistance to chloramphenicol and nalixid acid 0%. In the conventional breeding we determined these strains: *Klebsiella oxytoca*, *Serratia odorifera* bv. 1, *Serratia odorifera* bv. 2 and *Citrobacter braakii*, which were isolated from cheese samples and *Escherichia coli,* which was isolated from bryndza and parenica samples. All of this microorganisms were resistant to ampicillin and susceptibile to chloramphenicol and nalixid acid. In the ecological breeding we determined these strains: *Raoultella ornithinolytica* resistant to ampicillin, *Serratia rubidaea* multiresistant to all of used antibiotic, which were isolated from milk samples and *Klebsiella oxytoca* resistant to ampicillin, *Klebsiella pneumoniae* resistant to ampicillin and *Escherichia coli* resistant to ampicillin, which were isolated from cheese samples. On the basic of variation-statistical calculations, we determined the basic variation-statistical values like average, standard deviation, coefficient of variation, minimum, maximum and frequency of the size of inhibition zones. The highest values of variation (41.21%) was in the ecological breeding, when we used ampicillin. Minimum and maximum were in a range of 6 to 19 mm and average of inhibition zone was 10.25 mm. The lowest values of variation was 12.57% in the conventional breeding at the nalixid acid with the values of minimum and maximum in a range of 18 to 27 mm and average of inhibition zone 21.45%. In the frequency of the size of inhibition zones was in conventional breeding at the ampicillin in the range of 5 to 11 mm. In the ecological breeding frequency of the size of inhibition zones at the ampicillin was in the range of 8 to 14 mm. At the using chloramphenicol was in the both cases frequency of the size of inhibition zones in range of 16 to 24 mm. At the nalixid acid in the ecological breeding was a frequency of the size of inhibition zones in range of 16 to 20 mm and in conventional breeding in range of 20 to 24 mm.

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Address

Ing. Lukáš Hleba, doc. Ing. Miroslava Kačániová PhD., Katedra mikrobiológie, Ing. Simona Kunová, PhD., Katedra hygieny a bezpečnosti potravín, Slovenská poľnohospodárska univerzita v Nitre, 949 76 Nitra, Slovenská republika, e-mail: lukas.hleba@gmail.com, miroslava.kacaniova@gmail.com, simona.kunova@gmail.com Mgr. Maciej Kluz, Katedra biotechnologii i mikrobiologii, Wydzial biologiczno-rolniczy, Ul. Cwiklinskiej 2, 35-601 Rzeszow, Poland, e-mail: kluczyk82@op.pl, Ing. Mgr. Martin Mellen, PhD. Ministerstvo pôdohospodárstva SR, Dobrovičova 12, 812 66 Bratislava, Slovenská republika, e-mail: artin.mellen@gmail.com