ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS
SBORNÍK MENDELOVY ZEMĚDĚLSKÉ A LESNICKÉ UNIVERZITY V BRNĚ

Volume LVI 13 Number 2, 2008

OCCURRENCE OF ENTEROCOCCI IN RAW PORK AND BEEF AND THEIR ANTIBIOTICS MULTIRESISTANCE

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Received: November 23, 2007

Abstract

KROČKO, M., ČANIGOVÁ, M., DUCKOVÁ, V.: *Occurrence of enterococci in raw pork and beef and their antibiotics multiresistance*. Acta univ. agric. et silvic. Mendel. Brun., 2008, LVI, No. 2, pp. 101–106

The aim of this study was to determine the microbial contamination of raw pork and beef, to estimate the prevalence of enterococci and investigate the antibiotic multiple resistance of enterococci. Total bacterial counts (TBC) were cultured on Plate count agar and enterococci count were cultured on Slanetz – Bartley agar. The TBC after 24 h *post mortem* reached the value 3.61 ± 0.78 log cfu.cm^{-2} for pork and 2.58 ± 0.63 log cfu.cm^{-2} for beef. The count of enterococci after 24 h *post mortem* reached the value 1.98 ± 1.29 log cfu.cm^{-2} for pork and 1.16 ± 0.47 log cfu.cm^{-2} for beef. The average value of TBC in pork and beef were significantly ($P < 0.05$) higher after 7 days of ripening at 4 °C storage than 24 h *post mortem* and in pork and beef reached the value 4.69 ± 1.46 log cfu.cm^{-2} and 4.32 ± 1.44 log cfu.cm^{-2} resp. The average values of enterococci count after 7 days of ripening in pork and beef were 2.00 ± 1.27 log cfu.cm^{-2} and 0.84 ± 0.80 log cfu.cm^{-2} resp. Susceptibilities of isolated enterococci from pork to antimicrobial agents were tested using the disc diffusion method. *Enterococcus faecium* was the predominat species out of 50 isolates recovered from pork (72%), followed by *E. faecalis* (10%). Other enterococcal isolates were identified sporadically (*E. mundtti*–8%, *E. spp.*–10%). Out of 50 isolates of enterococci 14% were resistant to vancomycin and 10% were resistant to erythromycin, 18% to ampicillin, 24% to gentamicin and 34% to tetracycline. The calculated antibiotic code profiles indicated that large proportion of enterococci were resistant to all tested antibiotics except vancomycin. Our study suggests that raw pork and beef play a potential role as reservoirs of enterococci multiresistant to antibiotics.

raw beef, raw pork, enterococci, antibiotic resistance

Enterococci are common components of the gut microfloral community of mammals, birds, insects, and reptiles and are commonly found in soil, on plants, and in water. These microorganisms are particulary challenging to eliminate because of their ability to adapt to environmental stresses. Thus, it is not suprising that antimicrobial-resistant variants of enterococci have been recovered from meats, dairy products, and ready-to-eat foods and have even been found within probiotic formulations (Giraffa, 2002). Meat is exposed to microbiological risk due to its chemical-physical characteristics and the processing steps employed (Pizzin et al., 1998). The quality and shelf life of raw meat is determined by the growth of microorganisms. Storage of meat in chilling conditions provide the advantage to psychrotrophic bacteria. Some strains of enterococci are also able to survive the chilling conditions of meat ripening. Multiple-drug-resistant strains of *E. faecalis* and *E. faecium* have been increasingly associated with nosocomial infections. Of particular interest has been the potential for foods as a vehicle for transmission of these strains to humans (Sørensen et al., 2001). Indeed, there is strong epidemiological evidence to link the use of antibiotics in human medicine and farm animals with the presence of resistant strains in animal products. In general, the prevalence of antibiotic-resistant enterococci in farm animals and meat products is high (>60%) (Giraffa, 2002). It is well known that subtherapeutic use of antibiotics in the mass production of livestock may be responsible for the emergence and maintance of multiple antibiotic-resistant pathogenic bacteria (D´Aoust et al., 1991). The transfer of these microorganisms that are

resistant to antibiotics via the food chain from animals to humans is of increasing concern.The aim of this study was to evaluate the microbial contamination of raw pork and beef, estimate the prevalence of enterococci and investigate the antibiotic multiple resistance of enterococci.

MATERIAL AND METHODS

Samples for microbiological examination (95 samples) of pork (n = 70) and beef (n = 25) were taken from animals originated from four farms. The swine were processed in three different slaughterhouses with different sanitary and pre-slaughtering conditions. The cattle were processed in one slaughterhouse. Samples of pork (1.5 kg) and beef (2.5 kg) was carried out after 24 h *post mortem*. The surface swabs of pork were taken from the area of 25 cm^2 of thigh (*musculus semimembranosus*) and the surface swabs of beef were taken from the area of 25 cm^2 of saddle (*musculus longissimus dorsi*) (STN ISO 3100–2, 1999). After 7 days of pork (1.5 kg) and beef (2.5 kg) ripening at 4 °C were again taken swabs for the microbiological analysis.

Total mesophilic bacterial count (STN EN ISO 4833) and count of enterococci. The samples for enumeration of total mesophilic bacterial count were cultured on diagnostic Plate Count Agar (*HiMedia Laboratories*, India). Samples were incubated at temperature 30 ± 1 °C for 72 ± 2 h (STN ISO 4833, 2004). Enterococci were enumerated on Slanetz – Bartley agar at the temperature 37 °C for 48 ± 2 h (*Biokar Diagnostic,* France).

Isolation and species identification of enterococci. Suspect colonies of *Enterococcus* spp. derived from pork were examined as previously reported by Kročko et al. (2007).

Antimicrobial susceptibility tests. Before resistance tests, the isolates were resuscitated on Plate count agar (*HiMedia Laboratories*, India) at 30 ± 1 °C for 24 h. Inoculum was prepared by suspending of growth colonies from Plate count agar and the suspension was adjusted to equal a 0.5 McFarland standard according to the recommendations of National Committee for Clinical Laboratory Standarts (NCCLS, 1999). Susceptibilities to antimicrobial agents were tested using the disc diffusion method according to the NCCLS requirements, using the following antimicrobial discs: Vancomycin (VAN) 30 µg/disc, Gentamicin (GEN) 10 µg/disc, Erythromycin (ERY) 15 µg/disc, Tetracycline (TET) 30 µg/disc, Ampicillin (AMP) 10 µg/disc (*HiMedia Laboratories*, India). The isolates were classified as susceptible, intermediate resistant or resistant according to the NCCLS (1999) requirements.

Multiple antibiotic resistance. Multiple antibiotic resistance code profile according to Manie et al. (1998) was used. The antibiotics were divided into two groups and each antibiotic was designated a particular number. Group 1, which contained vancomycin, was designated number 1, tetracycline was designated number 2 and ampicillin was designated number 4; group 2 contained gentamicin and was also designated number 1 and erythromycin was desiganted number 2. If an isolate was resistant to a particular antibiotic it was given the number designated to that particular antibiotic. If the isolate was sensitive to the antibiotic it was given zero. The numbers awarded in the two groups were added to yield the respective code. For example, an isolate resistant to tetracycline, ampicillin and gentamicin but sensitive to the other antibiotics would receive the code (0 + 2 + 4) (1 + 0) to given a profile of 61 (Tab. I).

I: *Dividing of antibiotics in groups and its antibiotic code*

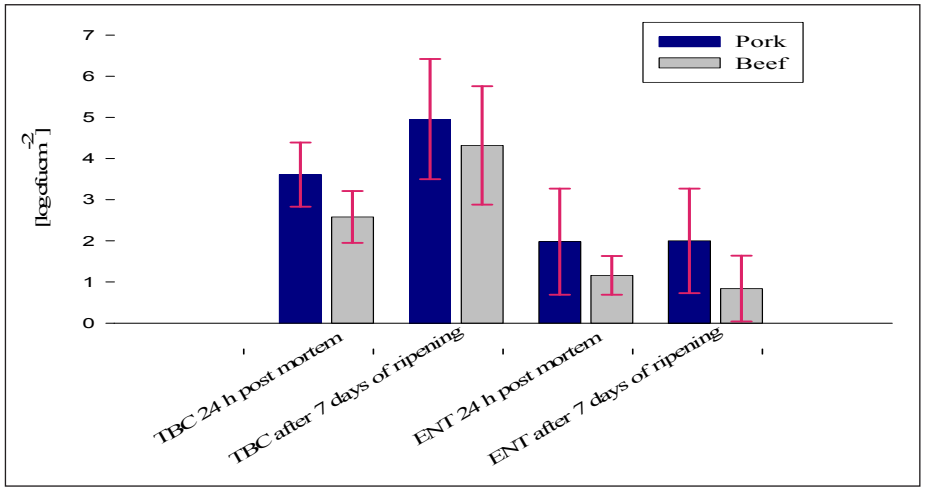
Group	1			2	
Antibiotics	vancomycin	tetracycline	ampicillin	gentamicin	erythromycin
Code	1	2	4	1	2

Statistical analysis. Microbiological data were transformed into logarithms of number of colony forming units (cfu.cm^{-2}) and were subjected to analysis of variance (Anova Single Factor). Means and standard deviations were calculated. When P-values were significant at the 0.05 level, mean differences were significant (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

The total mesophilic bacterial counts and counts of enterococci on surface pork and beef swabs, 24 h *post mortem* and after 7 days of ripening, are shown in Fig. 1.

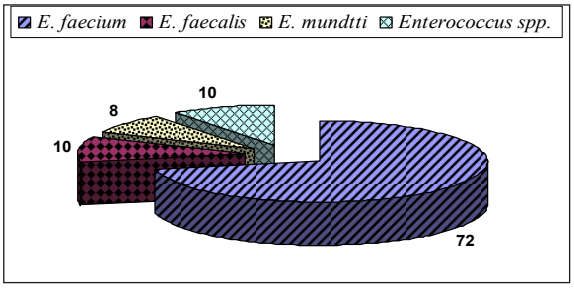
In general, mean values of the counts of investigated microbial groups 24 h *post mortem* on pork surface were 3.61 ± 0.78 log cfu.cm^{-2} for the total mesophilic bacterial count and 1.98 ± 1.29 log cfu.cm^{-2} for the enterococci. The mean values of total mesophilic bacterial counts and counts of enterococci on beef surface 24 h *post mortem* were 2.58 ± 0.63 log cfu.cm^{-2} and 1.16 ± 0.47 log cfu.cm^{-2}, respectively. The differences between the total mesophilic bacterial counts on pork surface from three abattoirs were statistically significant ($P < 0.05$), which could be explained by different pre-slaughter factors (transport distance, stabling conditions, shower), scalding and evisceration technique, sanitary and chilling conditions of abattoirs. Similary, the differences among enterococci counts on pork surfaces from three abattoirs were statistically significant ($P < 0.05$). The results are in agreement with findings of Zweifel et al. (2005), who reported significant differences among five Swiss



1: *The total bacterial count (TBC) and count of enterococci (ENT) in swabs from the surface area of beef and pork 24 h post mortem and after 7 days of ripening*

abattoirs ($P < 0.05$) and mean value of the total aerobic mesophilic bacterial counts from cattle carcasses ranged from 2.1 to 3.1 log cfu.cm^{-2} and from pig carcasses from 2.2 to 3.7 log cfu.cm^{-2}. In comparison, Mayr et al. (2003) found higher counts of aerobic mesophilic bacteria on the beef surface (5.13 log cfu.g^{-1}) and on the pork surface (4.74 log cfu.g^{-1}). Furthermore, authors found higher counts of enterococci in both beef and pork (3.81 log cfu.g^{-1}, 3.29 log cfu.g^{-1} respectively). Knudson and Hartman, (1993) reported that pig carcasses from three different slaughtering plants contained mean counts of enterococci 10^4–10^8 per 100 cm^{-2} of carcass surface.

The mean value of the total mesophilic bacterial count on pork and beef surface of the present experiment were significantly higher ($P < 0.05$) after 7 days of ripening at 4 °C storage than 24 h *post mortem* and in pork and beef surface reached the value 4.96 ± 1.46 log cfu.cm^{-2} and 4.32 ± 1.44 log cfu.cm^{-2} respectively. The mean values of enterococci count in pork and beef surface swabs were 2.00 ± 1.27 log cfu.cm^{-2} and 0.84 ± 0.80 log cfu.cm^{-2} respectively. The results indicated that total mesophilic bacterial count after 7 days of ripening in samples of pork increased by a 1.35 log cfu.cm^{-2} and in samples of beef by a 1.74 log cfu.cm^{-2}. In accordance with our results Pichner et al. (2000) found higher increase of aerobic mesophilic bacterial count (by 2–5 log series) after 7 days of ripening. Mayr et al. (2003) found a significant increase of the total aerobic mesophilic bacterial count and count of enterococci during cold storage in both beef and pork. They reported, that aerobic mesophilic bacterial count increased significantly after 2 to 3 days of storage at 4 °C, and maximum numbers were detected after 10 to 11 days. Furthermore, unlike our results, Mayr et al. (2003) found that the counts of *Enterococcus* spp. were significantly higher in beef than in pork. The counts of enterococci were significantly different ($P < 0.05$) between beef and pork after 7 days of ripening in our experiment. After 7 days of pork and beef storage at 4 °C under air conditions, the counts of *Enterococcus* spp. were significantly higher ($P < 0.05$) in pork than in beef. The decrease of enterococci count on a beef surface were not statisticaly significant ($P > 0.05$). Potentialy high contamination of meat with enterococci occurs already during slaughter (Witte, 2000) and during the processing of raw meat (Son et al., 1999; Schlegelová et al., 2004). The shelf-life of raw pork and beef depends greatly on the initial microbial contamination. Because of their high heat tolerance and survival under adverse environmental conditions, enterococci can colonise diverse foodstuffs and may then serve as indicators of the sanitary quality of food. Mainly *E. faecium* (72%) and *E. faecalis* (10%) were identified among the 50 enterococci isolates of pork surface swabs in the present experiment. Other enterococcal isolates were identified sporadically (*E. mundtti*–8%, *E. spp*. 10%) (Fig. 2). Enterococci from beef surface were not submited to the identification.

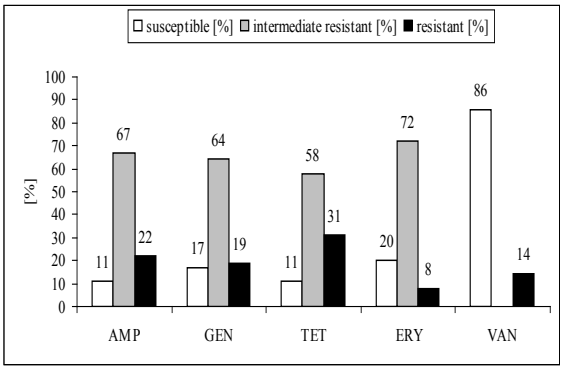


2: *The occurrence of enterococci [%] in swabs from the surface area of pork*

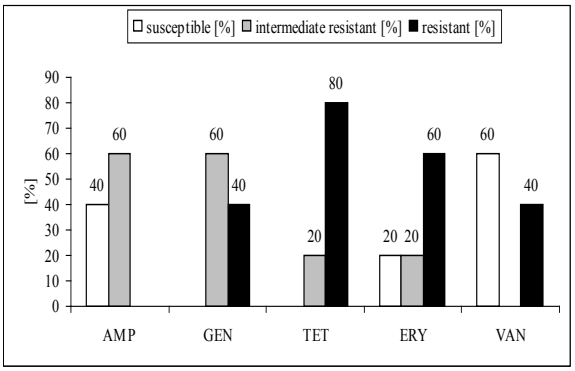
E. faecium and *E. faecalis* were the most frequently isolated species (35.6 and 33.3%, respectively) from up to 45% samples of various kinds of raw meat, including beef, collected from the retail trade in work of Pavia et al. (2000). Hayes et al. (2003) found that *E. faecium* was the predominant species recovered

from ground turkey (60%), ground beef (65%), and chicken breast (79%), while *E. faecalis* was the predominant species recovered from pork (54%).

Out of 50 isolates of enterococci from pork surface swabs, 14% were resistant to vancomycin and 10% were resistant to erythromycin, 18% to ampicillin, 24% to gentamicin and 34% to tetracycline. Significantly higher ($P < 0.05$) prevalence of intermediate resistant isolates from pork surface swabs to ampicillin (70%), gentamicin (66%), tetracycline (54%) and erythromycin (64%) were detected. Among the isolates this types of resistance were detected mainly in *E. faecium*. Resistance to antibiotics of *E. faecalis* and *E. faecium* performed by agar disc diffusion method is shown in Fig. 3 and Fig. 4. Resistance to vancomycin was detected in both *E. faecium* (14%) and *E. faecalis* (40%).



3: *Antimicrobial resistance profiles of Enterococcus faecium isolated from pork*



4: *Antimicrobial resistance profiles of Enterococcus faecalis isolated from pork. AMP–ampicillin, GEN–gentamicin, TET–tetracycline, ERY–erythromycin, VAN–vancomycin.*

Gambarotto et al. (2001) isolated 10.2% vancomycin resistant *E. faecium* strains and no *E. faecalis* strain from pork and poultry samples. In comparison, Lemcke and Bülte (2000) found 10% vancomycin resistant *E. faecium* and 10% vancomycin resistant *E. faecalis* in pork. Other European studies have observed vancomycin resistant enterococci (VRE) contamination in meat, from 8.3% of meat samples in Germany (Klein et al., 1998) to 79% of poultry samples in the Netherlands (Van den Braak et al., 1998). The isolates of *E. faecium* and *E. faecalis* were the most resistant to tetracycline in the present experiment. This may be attributed to subtherapeutic doses of tetracycline being used on pork farms which select for resistant strains, despite the widespread belief to the contrary. In study by Levy (1984), the effect of tetracycline-laced feed on the gut flora of chickens was demonstrated in two groups of 150 chickens raised with or without tetracycline in their feed. Within 36–48 h, virtually all enterics isolated from chickens in the tetracycline-treated cages were resistant to the drug. Within 3 months, resistance to tetracycline was accompanied by resistance to ampicillin, carbenicillin and sulphoamides. This rise in multiple resistance was accompanied by an increased ability of these strains to transfer tetracycline resistance. Also Chopra and Roberts (2001) reported that high level of resistance to tetracycline in isolates of pork is likely related to the wide use of this class of antibiotics in husbandry activities.

Significantly ($P < 0.05$) higher level of resistance to gentamicin, tetracycline, erythromycin and vancomycin was detected in *E. faecalis* than in *E. faecium* isolates. Contrary to the above finding the intermediate resistance to ampicillin, gentamicin, erythromycin and tetracycline were more prevelant in *E. faecium* isolates. Aarestrup et al. (2002) compared the levels of occurrence of antimicrobial resistance among *E. faecalis* and *E. faecium* isolates from pigs in Denmark, Spain and Sweden. In comparison with our results they found more frequent occurrence of resistance to erythromycin in isolates from Denmark (*E. faecium* 81%, *E. faecalis* 85%) and Spain (86% *E. faecium*). Among *E. faecium* isolates, the highest frequency of resistance was found among isolates from Spain and Denmark. Only in *E. faecium* isolates the authors detected resistance to vancomycin. More frequent resistance to tetracycline in *E. faecium* isolates derived from pigs in Denmark and Spain was also detected in the above mentioned experiment (Aarestrup et al., 2002).

The transfer via the food chain from animals to humans of microorganisms that are resistant to antimicrobial agents is of increasing concern. Recent data suggest that most enterococci have naturally inherent resistance and after exposure to antibiotics acquired resistance to various drugs (Hodges et al., 1992). Our results proved that isolates from pork surface swabs (96%) showed multi-resistence to two or more antibiotics. Isolates exhibiting intermediate resistance were recorded as resistant (Table II).

The results indicated that a large proportion of the enterococci from pork surface was resistant to a variety of the antibiotics tested. The antibiotic code profile 63 indicated that large proportion of enterococci were resistant to all tested antibiotics except vancomycin. The most frequently isolated multiresistant strain in the present experiment was *E. faecium*.

In work of Johnston and Jaykus (2004), 61% of *E. faecium* isolates and 11% of *E. faecalis* isolates showed multidrug resistance to 17 different antibiotics, although no specific patterns of multidrug resistance were readily apparent. High frequency of resistance to tetracycline and erythromycin was observed in all the groups of strains from swine, poultry and human in work of Busani et al. (2004).

II: *The antibiotic code profile of enterococci*

Multiple resistant profiles of *Enterococcus* spp. [%]										
Enterococcus (E.)	code									
	22	23	41	43	60	61	62	63	71	73
E. faecium	2	2		4	2	6	4	38	2	8
E. faecalis		4						2	2	2
E. mundtti			4			2		2		
E. spp.						4		6		

The abbreviations of codes are explained in chapter Material and Methods

SOUHRN

Výskyt enterokoků ve vepřovém a hovězím mase a jejich multirezistence na antibiotika

Výskyt multirezistentních enterokoků k antibiotikům v syrovém mase a masných výrobků a rovněž přenos těchto mikroorganismů potravovým řetězcem má vzrůstající důležitost. Náš výzkum prokázal, že syrové vepřové a hovězí maso je zdrojem enterokoků a zrecí proces v délce sedm dní v podmínkách chladírenského uskladnění (4°C) neumožňuje jejich průkazný pokles. Testováním rezistence enterokoků k antibiotikům bylo zjišteno, že většina izolovaných enterokoků byla rezistentní ke všem použitým antibiotikům (gentamicin, erythromycin, tetracycline and ampicillin) kromě vankomycinu. Vysoká úroveň rezistence u izolátů enterokoků z vepřového masa k tetracyklínu pravděpodobně souvisí se širokým použitím této skupiny antibiotik v zemědělské činnosti.

syrové hovězí, vepřové, enterokoky, antibiotická rezistence

SUMMARY

The occurrence of antibiotic multiresistant enterococci in raw meat and meat products, as well as transfer of these microorganisms via the food chain to humans is of increasing concern. Our investigation showed that raw pork and beef is source of enterococci and ripening process of meat in chilling conditions (4°C) during 7 days, not allow a significant decrease of these microorganisms. The calculated multiresistant code profiles in isolates of pork indicated that mainly *E. faecium* and *E. faecalis* were resistant to all tested antibiotics (gentamicin, erythromycin, tetracycline and ampicillin) without vancomycin. The high level of resistance to tetracycline in enterococci isolates of pork is likely related to the wide use of this class of antibiotics in husbandry activities.

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