ACTA SCIENTIARUM POLONORUM *Acta Sci. Pol., Zootechnica 11 (2) 2012, 43–50*

IMPACT OF PROBIOTICS ON NUMBERS, ABILITY TO HEMOLYSIS AND ON CHANGES IN DRUG-RESISTANCE OF SELECTED *ESCHERICHIA COLI* ISOLATES RECOVERED FROM FAECES OF DAIRY CATTLE

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Abstract. The investigations were conducted on 60 Holstein-Friesian dairy cows (at age 3 year and weight 590 kg) kept in tie-stall barn. The animals were divided into 3 groups of 20 heads each. The control group (K) was fed diets without probiotics, group (EM) – was fed diet with the addition of EM probiotic (dose of 150 ml × t^{-1} TMR) and group (T) – was fed diet with the addition of ToyoCerin probiotic (dose of 0.2 kg × t^{-1} TMR). Strains of *Escherichia coli* were isolated from faeces with the aim to determine their numbers, capability for hemolysis and assessment of their drug-resistance. The isolates were identified as *E. coli* on the basis of their biochemical properties API 20E (BioMérieux) and the PCR method. When analysing the capability of *Escherichia coli* for hemolysis, the highest number of haemolytic strains was determined in the faeces of animals fed diets with the addition of the EM probiotic. The examined isolates were characterised by different degrees of resistance to the antibiotics used in experiments. The smallest ($P<0.05$) number of resistant isolates was determined in group T.

Keywords: drug-resistance, *Escherichia coli*, hemolysis, probiotics

INTRODUCTION

Together with the imposition of a ban on the application of antibiotic growth stimulators, probiotics find a growing application in animal feeding and prophylaxis [Rekiel 2008]. In their composition, they can comprise lactic bacteria as well as other bacteria, e.g. *Bacillus* sp. These preparations are necessary for the life of animals as well as for their proper development. Introduced into diets, they can assist processes of digestion as well as nutrient absorption and exert a beneficial impact on the general health of animals. Lactic acid rods are characterised by strong antagonistic properties. This feature can be conditioned by the production by them of organic acids reducing pH (lactic, acetic), bacteriocins of bacteriostatic and bactericidal properties and release of hydrogen peroxide (H_2O_2)

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[Pericone et al. 2000, Sookkhee et al. 2001]. Hydrogen peroxide produced by *Lactobacillus* genus in quantities exceeding thresholds toxic for pathogens [Adesokan et al. 2010]. The appropriate quantitative and qualitative composition of lactic rods can influence homeostasis of the gastrointestinal tract and, in cases when application of antibiotics turns out to be necessary, it can enhance recovery of animal. The quantitative and qualitative equilibrium of the gastrointestinal tract can be upset by stress factors, for example temperature fluctuations, changes in feed composition, nutritional errors as well as the presence of enterotoxins produced by pathogenic intestinal microflora [Janik et al. 2006]. The most important intestinal biota pathogens comprise: *Escherichia coli* and *Salmonella* spp. [Casey et al. 2004] which can exhibit high drug resistance. Bearing in mind threats posed to humans and animals (transfer of resistant pathogens) by antibiotic resistant bacterial strains, it is essential to carry out investigations on possible impact of probiotic preparations on changes in drug resistance in selected pathogenic bacteria [Osek 2003].

The aim of the performed investigations was to determine the effect of two probiotic preparations (containing in its composition for example lactic acid bacteria, yeasts and bacilli) on the numbers and changes in drug resistance as well as on the ability for hemolysis of *Escherichia coli* bacteria isolated from animal faeces.

MATERIAL AND METHODS

A study was conducted on 60 Holstein-Friesian dairy cows (at age 3 year and weight 590 kg) kept in tie-stall barn. The animals were divided into 3 groups of 20 heads each. The control group (K) was fed diets without supplementation with probiotics, group (EM) – was fed diet with the addition of EM probiotic (dose of 150 ml × t^{-1} TMR), group (T) – was fed diet with the addition of ToyoCerin probiotic (dose of 0.2 kg × t^{-1} TMR). TMR contained: 55% maize silage, 10% grass silage (*Lolium multiflorum* L.), 25% barley and 10% extracted soybean meal (dose calculated to yeild 40 kg of milk per day).

Probiotics

EM-probiotic (Greenland Technologia EM): total number of *Lactobacillus casei Lactobacillus plantarum* lactic acid rods – 5×10^6 cfu × ml^{-1}; total number of *Saccharomyces cerevisiae* yeast cells – $5x10^3$ cfu × ml^{-1} and *Rhodopseudomonas palustris*.

Toyo Cerin (distributor VitTra Polska): spores of *Bacillus cereus var. toyoi* 10^9 cfu × g^{-1}; carrier (calcium carbonate) of 39.0–39.5% Ca content.

Performed analyses

Escherichia coli isolation was performed on McConkey (Merck) medium with incubation time – 48 h at 37ºC. A sample of 10 g faeces (taken from the rectum, 30 days after the application of probiotics) was added to 90 ml physiological salt and homogenised for 20 minutes (Stomacher Smasher, AES Chemunex, France). Cultures were made from consecutive dilutions from 10^5 to 10^{12}. The developed colonies were tested in the direction of biochemical properties using API 20E (BioMérieux) and PCR method for the presence of the universal stress protein gene (*uspA*). Starter sequences used for the PCR [Siegele 2005] are presented in Table 1.

Table 1. PCR primer used in the study
Tabela 1. Markery PCR użyte w doświadczeniu

Target bacteria Bakteria docelowa	Primer code Kody starterów	Primer sequence (5'→3') Sekwencja starterów (5'→3')	Target gene Wykrywany gen	PCR amplicon (bp) Wielkość produktu (pz)
Escherichia coli	Ec1 Ec2	5' CCG ATA CGC TGC CAA TCA GT 3' 5' ACG CAG ACC GTA GGC CAG AT 3'	*uspA*	884

In order to assess the resistance of the isolates, the disc method was employed using the following antibiotics (Oxoid): amoxicillin (AMX 25 μg), ampicillin (AMP 10 μg), gentamycin (GM 10 μg), neomycin (N 30 μg) and streptomycin (STR 10 μg). Culturing was conducted on nutrient broth (NB Merck) which, after 18 hours of incubation at the temperature of 37°C, was diluted at 1:10,000 in sterile physiological liquid. The suspension (500 μl each) was screened onto plates with Mueller-Hinton (Oxoid) substrate and discs with antibiotics were placed on the agar surface [CLSI 2002]. Following 18-hour incubation at 37°C, inhibition zone diameters were determined. The control of antibiotic activity was carried out with the assistance of the *Escherichia coli* 10,198 reference strain (DSMZ).

The ability of bacteria for hemolysis was assessed on an agar substrate supplemented with 5% ram blood. The incubation was carried out for 18–24 h at 37°C. Occurrence of a lighter zone surrounding the colony was considered as a positive result.

Results of investigations regarding numbers of microorganisms were subjected to statistical analysis using the *glm* procedure of the SAS program [1999] and the significance of differences was verified by Duncan's test.

RESULTS AND DISCUSSION

Using the API 20E set, the examined 60 isolates were classified as *Escherichia coli*. *Escherichia coli* strains isolated from faeces were subjected to PCR analysis in the direction of determining the presence of fragments of the *uspA* gene. The amplification with the assistance of the PCR method revealed that all the examined isolates (n = 60) had the capability to manufacture the amplicons of the 884 bp mass typical for *Escherichia coli* (Photo 1). Mean numbers of cfu $\log_{10}$ (n = 20 for each combination) and frequency of the occurrence of bacteria isolated from saliva and faeces of experimental cows are collated in Table 2.

Escherichia coli were determined in each examined sample of faeces (n = 60). In groups K and EM, counts of these bacteria were similar and did not differ statistically significantly ($P<0.05$). The lowest ($P<0.05$) numbers of *Escherichia coli* were determined in group T. Capability to cause hemolysis by *Escherichia coli* isolates (Table 3) revealed that the highest number of isolates with haemolytic traits was recorded in EM experimental group (60%).

High numbers of haemolytic isolates were determined in the faeces of animals fed diets with the addition of EM probiotic, the lowest in combination T.

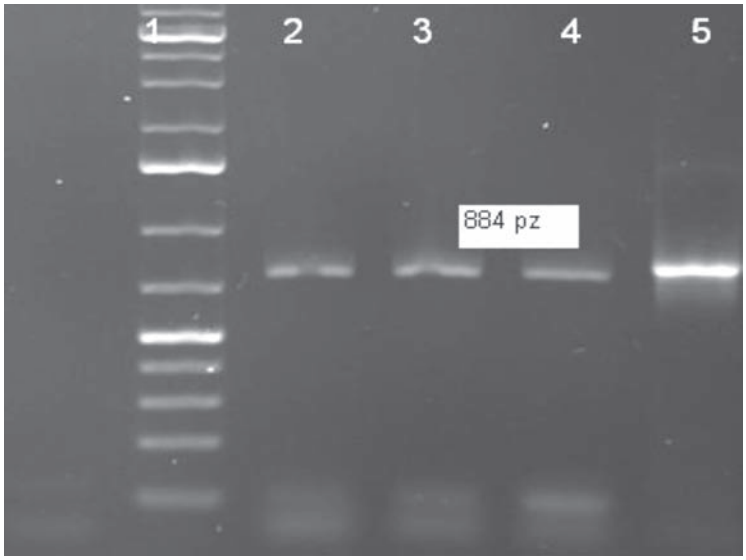

Photo 1. Separation of PCR products in 1.5% agarose gel of the uspA gene fragment (884 bp) specific for *Escherichia coli*

Fot. 1. Rozdział produktów PCR fragmentu genu uspA (884 pz) w 1,5-procentowym żelu agarozowym specyficznym dla *Escherichia coli*

Table 2. Frequency of occurrence and numbers of the examined bacteria in faeces of the examined cows (n = 60)

Tabela 2. Częstotliwość występowania oraz liczebność badanych bakterii w kale krów (n = 60)

Target bacteria Bakteria docelowa	Combinations – Kombinacje		
	K (n = 20)	EM (n = 20)	T (n = 20)
Escherichia coli Frequency incidence Częstotliwość występowania	20/20 (100%)	20/20 (100%)	20/20 (100%)
CFU $\log_{10}$ ml^{-1} faeces – kału	3.17a	3.22a	1.99b

a, b, – means in rows designated with the same letters do not differ significantly at the level of P<0.05.
a, b, – średnie w rzędach oznaczone tymi samymi literami nie różnią się istotnie na poziomie P<0,05.

Table 3. Numbers and percentages of *Escherichia coli* isolates ability for hemolisis

Tabela 3. Liczebność i procent izolatów *Escherichia coli* zdolnych do hemolizy

Hemolisis Hemoliza	Combinations – Kombinacje		
	K (n = 20)	EM (n = 20)	T (n = 20)
Hem β	3 (15%)	12 (60%)	1 (15%)

Table 4 collates results concerning the resistance of *Escherichia coli* isolates to selected antibiotics. The examined isolates were characterised by different degree of resistance against antibiotics applied in the described experiments. Numbers and proportions of

resistant isolates in groups K and EM were similar (P<0.05). The smallest number of resistant isolates was determined in group T. In all combinations reported the highest percentage of resistance to neomycin and streptomycin.

Table 4. Antibiotic resistance of *Escherichia coli* isolates (n = 60)
Tabela 4. Oporność *Escherichia coli* na antybiotyki (n = 60)

Antbiotics Antybiotyki	Combinations – Kombinacje		
	K (n = 20)	EM (n = 20)	T (n = 20)
Amoxycilline (AMX 25 μg) Amoxycilina	11a (55%)	10a (50%)	8b (40%)
Ampicillin (AMP 10 μg) Ampycylina	10a (50%)	11a (55%)	9a (45%)
Gentamicin (GM 10 μg) Gentamycyna	2a (10%)	3a (15%)	1b (5%)
Neomicin (N 30 μg) Neomycyna	18a (90%)	17a (85%)	10b (50%)
Streptomycin (STR 10 μg) Streptomycyna	19a (95%)	18a (90%)	11b (55%)

a, b, – means in rows designated with the same letters do not differ significantly at the level of P<0.05.
a, b, – średnie w rzędach oznaczone tymi samymi literami nie różnią się istotnie na poziomie P<0,05.

The gastrointestinal tract of animals can be inhabited by over 200 different types of bacteria. The colonisation velocity occurs gradually and depends on zoohygienic conditions, feed composition and its consumption as well as on the age of animals [Khunajakr et al. 2008]. When analysing biological and biochemical mechanism of action of probiotic preparations, it is important to pay attention to the functions they play in animal organisms. Lactic acid bacteria constituting part of the composition of these preparations can manufacture various antibacterial substrates such as: organic acids, bacteriocins and hydrogen peroxide [Barbosa 2000, Rial 2000]. It should be remembered that the effectiveness of bacterial action exhibiting probiotic traits depends on interactions between other aerobic and anaerobic bacteria settling the feed and gastrointestinal tract of animals [Otero et al. 2006].

Probiotic preparations can contain in their composition also bacteria from the *Bacillus* genus capable of manufacturing enzyme from the group of amylases and proteases. Their action reduces production of ammonia and hydrogen sulphide in the gastrointestinal tract [Ito et al. 2003]. Furthermore, it should also be remembered that bacteria from *Lactobacillus* and *Bacillus* genera can produce different bacteriocins of bactericidal and bacteriostatic properties which can provide an alternative for antibiotic therapies [Cao et al. 2007]. Antibiotic growth promoters applied earlier caused wall thinning of the gastrointestinal tract (especially intestines) as well as their better blood supply and, hence, better nutrient absorption [Ternak 2006]. However, application of antibiotics also involved risks associated with the development and intensification of resistance in bacteria [Salisbury et al. 2002]. Transfer of many resistant pathogenic bacteria from animals to people and in the opposite direction turned out to be a very dangerous phenomenon. Veterinary

doctors, butchers and farmers are among the highest risk groups [Teuber 2001]. Therefore, attempts should be made to reduce drug resistance in *Escherichia coli* and the consumption of chemotherapeutics used for treatment and, simultaneously, to improve their effectiveness. In our own studies, when assessing haemolytic properties of the examined *Escherichia coli* isolates, considerable differences were observed in proportions of haemolytic strains. This trait is an important, indirect indicator of the pathogeneity of these bacteria. There is a significant correlation between the pathogenicity and the capability of developing *in vitro* hemolysis by *Escherichia coli* strains [Dacko et al. 2004]. An important phenotypic trait which can be applied as a basis for the classification and differentiation of pathogenic *Escherichia coli* strains is their '*in vitro*' resistance to commonly used chemotherapeutics [Selwet et al. 2010]. There are numerous recognised transfer mechanisms of resistance to antibiotics by bacteria. An important resistance mechanisms is antibiotic deactivation by enzyme, e.g. β-lactams which comprise approximately 340 enzymes. Appearance of β-lactamases of a wide substrate profile (ESβL) has become a serious epidemiological problem. ESβL genes can be easily transferred within related species of the *Enterobacteriaceae* family [Mazur and Klag 2004].

CONCLUSIONS

Recapitulating, the results obtained in this study confirm the need to carry out permanent investigations monitoring the impact of probiotic preparations on changes in the sensitivity of isolated bacteria to applied antibiotics. It should be remembered, however, that we talk about resistance to antibiotics when mean concentrations inhibiting *in vitro* bacterial populations are higher in comparison with concentrations possible to achieve *in vivo*.

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WPŁYW PROBIOTYKÓW NA LICZEBNOŚĆ, ZDOLNOŚĆ DO HEMOLIZY ORAZ ZMIANY LEKOOPORNOŚCI WYBRANYCH IZOLATÓW *ESCHERICHIA COLI* POZYSKANYCH Z KAŁU KRÓW MLECZNYCH

Streszczenie. Badaniami objęto 60 krów mlecznych rasy holsztyńsko-fryzyjskiej (w wieku trzech lat, o masie 590 kg) hodowanych w oborze uwięziowej. Zwierzęta podzielono na trzy grupy doświadczalne po 20 osobników. Grupa kontrolna (K) – żywiona bez dodatku probiotyku; grupa (EM) – żywiona z dodatkiem probiotyku EM (dawka 150 ml × t^{-1} TMR); grupa (T)

– żywiona z dodatkiem probiotyku ToyoCerin (dawka 0,2 kg × t^{-1} TMR). Szczepy *Escherichia coli* izolowano z kału w celu określenia ich liczebności, zdolności do hemolizy i określenia ich oporności na wybrane antybiotyki. Wyrosłe kolonie *Escherichia coli* testowano w celu określenia przynależności gatunkowej z zastosowaniem API 20E (BioMérieux) oraz metodą PCR. Badając zdolność *Escherichia coli* do hemolizy, największą ilość szczepów hemolitycznych oznaczono w kale zwierząt żywionych z dodatkiem probiotyku EM. Badane izolaty wykazywały zróżnicowanie pod względem oporności na użyte w badaniu antybiotyki. Istotnie najmniejszą ($P<0,05$) liczbę izolatów opornych oznaczono w grupie T.

Słowa kluczowe: *Escherichia coli,* hemoliza, lekooporność, probiotyki

Accepted for print – Zaakceptowano do druku: 12.05.2012