IMPACT OF PROBIOTIC ON THE NUMBER OF LACTIC ACID RODS FORMING HYDROGEN PEROXIDE ISOLATED FROM PORKERS AND ON CHANGES IN DRUG RESISTANCE OF SELECTED ESCHERICHIA COLI ISOLATES

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Abstract

The presented investigations were conducted on a group of 60 porkers of crossbreed Polish Landrace x Large White Polish. The animals were divided into two equal experimental groups. The control group (K) was fed diets without supplementation with probiotics, group (P) – diets with the addition of probiotic (0.2 kg t⁻¹ feed). The aim of the study was to determine the effect of probiotic preparation on total number of lactic acid rods from the Lactobacillus genus and those forming hydrogen peroxide. The second part of experiment concerned the influence of probiotic preparation on the number, haemolytic ability and changes in drug resistance of Escherichia coli isolated from animal faeces. The significantly highest number of Lactobacillus sp. were determined in the saliva of porkers fed diets with the addition of probiotic, while the lowest in the control group. Lactobacillus sp. rods capable of forming hydrogen peroxide were isolated from 17 animals in group K and from three animals in group P. E. coli was determined in each examined sample of faeces. In groups K and P, counts of these bacteria were similar and did not differ statistically. High numbers of haemolytic isolates (haemolysis β) were found in faeces of animals fed diets with the addition of probiotic. Number and proportions of resistant isolates in groups K and P were different. Gentamicin was characterized by exceptionally high in vitro effectiveness. The used probiotic increased drug resistance of E. coli and increased frequency of incidence of haemolysis β.

Key words: Escherichia coli, Lactobacillus, probiotic, drug resistance, hydrogen peroxide.

In pig farming, antibiotics are generally used in suckling and weaned pigs for prevention of intestinal infection caused by enteric pathogens and for growth promotion (6). Together with the imposition of a ban on the application of antibiotic growth stimulators, probiotics find a growing application in animal feeding and prophylaxis (15, 20). Their composition can comprise lactic acid bacteria as well as other bacteria, e.g. Bacillus sp. These preparations are essential for the life of animals and for their proper development. Introduced into diets, they can assist processes of digestion as well as nutrient absorption, and can exert a beneficial impact on the general health condition of animals (19). Lactic acid bacteria introduced with feed or naturally settling the mouth can be represented by either streptococci or rods. Lactic acid rods are characterised by strong antagonistic properties, which can result from the synthesis of organic acids reducing pH (lactic, acetic), bacteriocins of bacteriostatic and bactericidal properties, and by release of hydrogen peroxide (H₂O₂) (14, 18). Hydrogen peroxide manufactured by certain bacterial strains of the Lactobacillus genus in quantities exceeding thresholds toxic for pathogens can act as a factor inhibiting their development (1). Furthermore, the compound exhibits a strong oxidising activity and impairs proteins in cell structures of the pathogens (21). The appropriate quantitative and qualitative composition of lactic acid rods can influence homeostasis of the gastrointestinal tract and, in cases when application of antibiotics turns out to be necessary, it can enhance recovery. 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 (8). The most important intestinal biota pathogens comprise Escherichia coli (2) and Salmonella sp. (3), 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 (12).
The aim of the performed investigation was to
determine the effect of probiotic preparation on the total
number of lactic acid rods from the *Lactobacillus* genus
and those forming hydrogen peroxide. The second part
of the experiment concerned the influence of probiotic
preparation on the number, haemolytic ability and
changes in drug resistance of *E. coli* isolated from
animal faeces.

**Material and Methods**

The presented investigations were conducted on
a group of 60 porkers of crossbreed Polish Landrace x
Large White Polish. The animals were divided into two
equal experimental groups: the control group (K) fed a
diet without probiotic and group (P) fed a diet with the
addition of probiotic (0.2 kg t⁻¹ feed).

**Probiotic.** Spores of *Bacillus cereus* var. *toyoi*
(10⁹ cfu g⁻¹); carrier (calcium carbonate) containing
39.0%-39.5% of Ca.

**Performed analyses.** Two millilitres of saliva
were collected to a sterile container from each animal. Then
1 ml of the saliva was diluted serially from 10⁻¹ to
10⁻⁹ in a sterile solution of physiological saline and 0.1
ml of the undiluted saliva and its consecutive dilutions
were spread with a sterile glass rod on the surface of
selective media (9). Rods of lactic acid were isolated
from consecutive dilutions from 10⁻⁵ to
10⁻¹² and incubated under anaerobic conditions (Anaerocult®/A Merck) at 37°C for 48 h. In
order to determine H₂O₂-forming lactic acid bacteria,
colonies developed on the Rogosa SL Agar were
transferred onto the Rogosa medium supplemented with
2.2'-azino-bis (3-ethylbenzotiazolino-6-sulfonic) acid
(Sigma) and peroxidase (Sigma). Lactic acid bacteria
capable of forming H₂O₂ developed in the form of
violet-coloured colonies. Lactic acid rods were
identified on the basis of biochemical traits using API
20A (BioMérieux), while those forming H₂O₂ were
additionally tested with the PCR method. The total
aerobic bacteria counts in saliva were determined on the
BHAgar with the addition of 5% sheep blood (BioMérieux) incubated for 48 h at 37°C, and anaerobic
bacteria counts – on Schaedler (BioMérieux) medium
incubated for 48 h at 37°C under anaerobic conditions
(Anaerocult®/A Merck).

*E. coli* isolation was performed on McConkey
(Merck) medium incubated for 48 h at 37°C. A sample
of 10 g of faeces was added to 90 ml of physiologic saline
and homogenised for 20 min (Stomacher Smasher, AES Chemunex, France). Cultures were made
from consecutive dilutions from 10⁻⁵ to
10⁻¹². The developed colonies were tested for biochemical
properties (to determine the biochemical profiles of *E.
coli*) using API 20E (BioMérieux) and PCR method for
the presence of the universal stress protein gene (*uspA*)
(5). Starter sequences used for the PCR are presented in
Table 1.

In order to assess the antibiotic resistance of the
isolates, the Kirby-Bauer disc method was employed
using the following antibiotics (Oxoid): amoxicillin (25
µg), ampicillin (10 µg), erythromycin (15 µg),
gentamycin (10 µg), neomycin (30 µg), and streptomycin (10 µg). Culturing was conducted on
nutrient broth (NB Merck), which was diluted at
1:10,000 in sterile physiological saline after 18 h of
incubation at 37°C. The suspension (500 µl each) was
screened onto plates with Mueller-Hinton substrate
(Oxoid) and the discs with antibiotics were placed on the
agar surface. Following 18 h incubation at 37°C, zones
of growth inhibition were determined. The control
of antibiotic activity was carried out with the assistance of the *Staphylococcus aureus* 209P reference strain.

The haemolytic ability of the bacteria was
assessed on an agar substrate supplemented with 5% sheep
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 the number of
microorganisms were subjected to statistical analysis
using the *glm* procedure of the SAS programme (16) and
the significance of differences was verified by Tuckey
test.

**Results**

The mean numbers of cfu log₁₀ and frequency
(%) of the occurrence of bacteria isolated from saliva
and faeces of experimental cows are presented in Table 2. The occurrence of *Lactobacillus* sp. rods was
confirmed in all examined animals in each experimental
group. The significantly (P<0.05) highest number of
*Lactobacillus* sp. was determined in the saliva of porkers
fed diet with the addition of probiotic, while the lowest
(P<0.05) in the control group. *Lactobacillus* sp. rods
were isolated from 17 animals in group K, and from three animals in group P. The highest (P<0.05) number of lactic acid rods forming
H₂O₂ were determined in group K and the lowest
(P<0.05) – in group P. Despite a small number of
individuals in group P from which rods capable of
forming H₂O₂ were isolated, also the number of these
bacteria was significantly lower (P<0.05) in comparison
with control group. *E. coli* was determined in each
examined sample of faeces. In groups K and P, counts of
these bacteria were similar.

The identified *Lactobacillus* sp. isolates
forming H₂O₂ were subjected to PCR analysis for the
presence of fragments of *recA*, *gyrB* 16S rRNA genes.
The PCR amplification allowed identification of the
following species in individual combinations: K (n=17) –
51% *Lactobacillus plantarum*, 26% *Lactobacillus buchneri*, and 23% *Lactobacillus brevis*; P (n=3) –
66.67% *Lactobacillus plantarum* and 33.33% *Lactobacillus buchneri*.

API 20E test allowed distinguishing two
biochemical profiles A and B (each profile showed the
same biochemical characteristics). Simultaneously, *E.
coli* isolated from faeces were subjected to PCR analysis
in order to determine the presence of the fragments of
the *uspA* gene. Amplification with the PCR method
revealed that all examined isolates (n=60) had the
capability to produce the amplicons of the 884 bp mass
typical for *E. coli*.
Table 1

<table>
<thead>
<tr>
<th>Target bacteria</th>
<th>Primer code</th>
<th>Primer sequence (5’→3’)</th>
<th>Target gene</th>
<th>PCR amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td>1P1 F</td>
<td>5’ TTT GAG TGA GTG GCG AAC TG 3’</td>
<td>recA</td>
<td>249</td>
</tr>
<tr>
<td></td>
<td>1P1 R</td>
<td>5’ CGT GTC TCA GTC CCA ATG TG 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus brevis</em></td>
<td>1P1 F</td>
<td>5’ GGA GTC AGG CGT CTA AGG 3’</td>
<td>gyrB</td>
<td>237</td>
</tr>
<tr>
<td></td>
<td>1P1 R</td>
<td>5’ ACG CAG TGG TCC GGT TT 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus buchneri</em></td>
<td>1P1 F</td>
<td>5’ GCG TCT CCG TTG ATG ATT TT 3’</td>
<td>16S rRNA</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td>1P1 R</td>
<td>5’ CCT AAA GTG ACA GCC GAA GC 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Ec1</td>
<td>5’ CCG ATA CGC TGC CAA TCA GT 3’</td>
<td>uspA</td>
<td>884</td>
</tr>
<tr>
<td></td>
<td>Ec2</td>
<td>5’ ACG CAG ACC GTA GCC CAG AT 3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Target bacteria</th>
<th>Combinations</th>
<th>K (n=30)</th>
<th>P (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus</em> sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency incidence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU log₁₀ ml⁻¹ saliva</td>
<td></td>
<td>30/30 (100%)</td>
<td>30/30 (100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.30a</td>
<td>5.75b</td>
</tr>
<tr>
<td><em>Lactobacillus</em> sp. producing H₂O₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency incidence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU log₁₀ ml⁻¹ saliva</td>
<td></td>
<td>17/30 (56.7%)</td>
<td>3/30 (10%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.99a</td>
<td>4.02b</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency incidence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU log₁₀ ml⁻¹ faeces</td>
<td></td>
<td>30/30 (100%)</td>
<td>30/30 (100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.50a</td>
<td>11.49a</td>
</tr>
</tbody>
</table>
a, b, – means in rows designated with the same letters do not differ significantly at the level of P<0.05
K-control group, P-group with probiotic

Table 3

<table>
<thead>
<tr>
<th>Haemolysis</th>
<th>Combinations</th>
<th>K (n=30)</th>
<th>P (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β</td>
<td>A (n=25)</td>
<td>4 (16%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td></td>
<td>B (n=5)</td>
<td>0 (0%)</td>
<td>2 (15.4%)</td>
</tr>
<tr>
<td>α</td>
<td>A (n=13)</td>
<td>21 (84%)</td>
<td>11 (84.6%)</td>
</tr>
<tr>
<td></td>
<td>B (n=7)</td>
<td>10 (58.8%)</td>
<td>7 (41.2%)</td>
</tr>
</tbody>
</table>
A, B-biochemical profil of *E. coli*

Table 4

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Combinations</th>
<th>K (n=30)</th>
<th>P (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxycilline 25 µg</td>
<td>9 (30%)</td>
<td>11 (36.7%)</td>
<td></td>
</tr>
<tr>
<td>Ampicillin 10 µg</td>
<td>8 (26.7%)</td>
<td>12 (40%)</td>
<td></td>
</tr>
<tr>
<td>Erythromycin 15 µg</td>
<td>12 (40%)</td>
<td>20 (66.7%)</td>
<td></td>
</tr>
<tr>
<td>Gentamicin 10 µg</td>
<td>3 (10%)</td>
<td>7 (23.3%)</td>
<td></td>
</tr>
<tr>
<td>Neomicin 30 µg</td>
<td>14 (46.7%)</td>
<td>22 (73.3%)</td>
<td></td>
</tr>
<tr>
<td>Streptomycin 10 µg</td>
<td>10 (33.3%)</td>
<td>25 (83.3%)</td>
<td></td>
</tr>
</tbody>
</table>
Determination of relationships between biochemical properties and capability to cause haemolysis by *E. coli* isolates (Table 3) revealed that the highest number of isolates capable of haemolysis was recorded in control group for profile A, and profile A, B in case of group P. High number of haemolytic isolates (haemolysis β) were determined in the faeces of animals fed diet with the addition of probiotic.

Table 4 collates the results concerning the resistance of *E. coli* isolates to selected chemotherapeutics. The examined isolates were characterised by different degree of resistance against the applied antibiotics.

The number and proportions of resistant isolates in groups K and P were different. Gentamicin was characterised by an exceptionally high effectiveness. Low bactericidal and bacteriostatic properties, which can also be remembered that bacteria from the group of amylases and proteases. Their action provide an alternative for antibiotic therapy (4).

In our studies, when assessing haemolytic properties of *E. coli* isolates, considerable differences were observed in proportions of haemolytic and non-haemolytic strains. This trait is an important indirect indicator of the pathogenicity of these bacteria. There is a significant correlation between the pathogenicity and the capability of developing in vitro haemolysis by *E. coli* strains (5). An important phenotypic trait, which can be applied as a basis for the classification and differentiation of pathogenic *E. coli* strains is their in vitro resistance to the commonly used chemotherapeutics (17). There are numerous recognised transfer mechanisms of resistance to antibiotics by bacteria. An important resistance mechanism is antibiotic deactivation by enzymes, 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 (10).

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.

**Discussion**

Hydrogen peroxide is among the important factors inhibiting the development of pathogens and preventing their penetration into the gastrointestinal tract of animals. H$_2$O$_2$ is produced by some strains of bacteria from the *Lactobacillus* genus in quantities exceeding toxicity thresholds. Adesokan et al. (1) recorded high production of H$_2$O$_2$ by *Lactobacillus brevis* and *Lactobacillus plantarum*. Among strains studied in vitro by these researchers, the *Leuconostoc mesenteroides* turned out to produce the smallest quantities of the compound. Different results were reported by Ogunbanawo (11), who demonstrated the highest in vitro production of H$_2$O$_2$ by *Leuconostoc mesenteroides*. In in vitro experiments on H$_2$O$_2$ production by different *Lactobacillus* sp., Zalán et al. (21) observed that *Lactobacillus plantarum* produced large quantities of H$_2$O$_2$ but this strain failed to exhibit inhibitory properties in relation to *E. coli*, although it inhibited growth of *Bacillus cereus* and *Listeria monocytogenes*. Results of many experiments demonstrated an inhibitory influence of H$_2$O$_2$ on such test strains as: *Staphylococcus aureus*, *Salmonella* sp., *E. coli* and *Listeria monocytogenes*. That is why it appears important to introduce probiotic preparations, which contain in their composition lactic acid bacteria capable of producing H$_2$O$_2$ into feeds (13). Probiotic preparations can also comprise bacteria from the *Bacillus* genus capable of producing enzymes from the group of amylases and proteases. Their action reduces production of ammonia and hydrogen sulphide in the gastrointestinal tract (7). 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 therapy (4).

In our studies, when assessing haemolytic properties of *E. coli* isolates, considerable differences were observed in proportions of haemolytic and non-haemolytic strains. This trait is an important indirect indicator of the pathogenicity of these bacteria. There is a significant correlation between the pathogenicity and the capability of developing in vitro haemolysis by *E. coli* strains (5). An important phenotypic trait, which can be applied as a basis for the classification and differentiation of pathogenic *E. coli* strains is their in vitro resistance to the commonly used chemotherapeutics (17). There are numerous recognised transfer mechanisms of resistance to antibiotics by bacteria. An important resistance mechanism is antibiotic deactivation by enzymes, 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 (10).

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.

**References**


