ACTA SCIENTIARUM POLONORUM *Acta Sci. Pol., Zootechnica 12 (3) 2013, 69–78*

IMPACT OF PROBIOTICS ON NUMBERS OF LACTIC ACID RODS PRODUCING HYDROGEN PEROXIDE ISOLATED FROM MOUTHS OF DAIRY CATTLE

Marek Selwet, Mariola Galbas, Filip Porzucek, Małgorzata Pociejowska, Tomasz Clapa

Poznań University of Life Sciences, Poland

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 $\cdot$ t^{-1} TMR) and group (T) – was fed diet with the addition of ToyoCerin probiotic (dose of 0.2 kg $\cdot$ t^{-1} TMR). The volume of 2–10 cm^3 saliva was collected from each animal in which the following parameters were determined: number of lactic acid rods from the *Lactobacillus* genus, number of rods capable of producing hydrogen peroxide. For purpose of precise diagnostics, lactic acid rods were identified on the basis of biochemical traits employing API 50 CHL (BioMérieux), while those manufacturing H2O2 were additionally tested using PCR method. The occurrence of *Lactobacillus* spp. rods was confirmed in all the examined individuals and in each and every experimental combination. *Lactobacillus* spp. rods capable of produce hydrogen peroxide were isolated in 17 cows in group K, in 3 individuals in group EM and in 13 animals in group T. EM probiotic strongly significantly restrict the development of *Lactobacillus* spp. strains are capable to produce hydrogen peroxide.

Key words: hydrogen peroxide, *Lactobacillus* spp., 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 acid bacteria as well as other bacteria, e.g. *Bacillus* spp. These preparations are necessary for life of

Corresponding author – Adres do korespondencji: dr hab. inż. Marek Selwet, Poznań University of Life Sciences, Department of General and Environmental Microbiology, ul. Szydłowska 50, 60-656 Poznań, Poland, e-mail: mselwet@jay.au.poznan.pl

animals as well as for their proper development. Introduced into diets, they can assist process of digestion as well as nutrient absorption and exert a beneficial impact on the general health of animals. Lactic acid rods are characterized 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 [Pericone et al. 2000, Sookkhee et al. 2001]. Hydrogen peroxide produced by *Lactobacillus* spp. in quantities exceeding thresholds toxic for pathogens [Adesokan et al. 2010]. 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 recover 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 performed investigations was to determine the effect of two probiotic preparations on total numbers of lactic acid rods from the *Lactobacillus* genus and those forming hydrogen peroxide isolated from the mouths of dairy cows.

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 $\cdot$ t^{-1} Total Mixed Ration), group (T) – was fed diet with the addition of Toyo Cerin probiotic (dose of 0.2 kg $\cdot$ t^{-1} TMR). TMR contained: 55% maize silage, 10% grass silage, 25% barley and 10% extracted soybean meal.

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 – 5×10^3 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

The total of 2–10 cm^3 of saliva was collected daily, after feeding for 10 days to sterile container from each animal. The 1 cm^3 of saliva was diluted serially from 10^{-1} to 10^{-9} in a sterile solution of physiological salt and 0.1 cm^3 saliva was taken from the undiluted sample and from its consecutive dilutions and it was spread with a sterile glass rod on the surface of selective media [Klebanoff et al. 1991]. Rods of lactic acid were isolated onto Rogosa SL Agar (Oxoid). Cultures were made from 10^{-1} to 10-^{3} dilutions and incubated in anaerobic conditions (Anaerocult A Merck) at the 37°C for 48 h. In order to determine hydrogen peroxide forming lactic acid rods, colonies developed on the Rogosa SL Agrar were transferred onto the Rogosa medium supplemented with 2.2'-azyno-bis (3-ethylobenzotiazolino-6-sulfonic) acid (Sigma) and peroxidase (Sigma). Lactic acid rods capable of produce hydrogen peroxide developed in the form of violet-colored colonies. Lactic acid rods were identified on the basis of biochemical traits using API 50 CHL (BioMérieux), while those forming H2O2 were additionally tested with the assistance of PCR method. Table 1 shows the PCR primers used in the study.

Table 1. Species-specific PCR primers used in the study

Tabela 1. Specyficzne dla badanych bakterii markery gatunkowe użyte w reakcji PCR

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
Lactobacillus plantarum	1P1 F 1P1 R	5' TTT GAG TGA GTG GCG AAC TG 3' 5' CGT GTC TCA GTC CCA ATG TG 3'	*recA*	249
Lactobacillus brevis	1P1 F 1P1 R	5' GGA GTC AGG CGT CTA AGG 3' 5' ACG CAG TTG CTC GGT TT 3'	*gyrB*	237
Lactobacillus buchneri	1P1 F 1P1 R	5' GCG TCT CCG TTG ATG ATT TT 3' 5' CCT AAA GTG ACA GCC GAA GC 3'	16S rRNA	193

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

RESULTS AND DISCUSSION

The most important biochemical traits were determined by API 50 CHL set making it possible to classify the examined isolates (capable of being cultured in the laboratory) as *Lactobacillus* spp. The occurrence of *Lactobacillus* spp. rods was confirmed in all examined animals each experimental treatment. *Lactobacillus* spp. rods capable of forming hydrogen peroxide were isolated from 17 animals in group K, from 3 animals in group EM and from 13 animals in group T (Table 2).

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

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

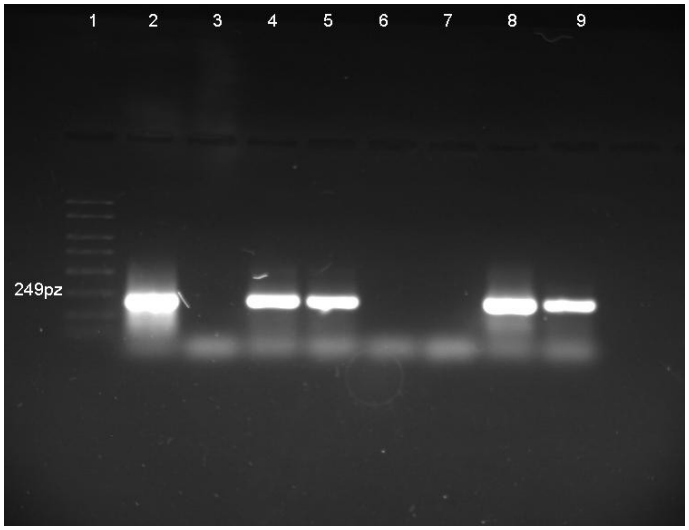
Target bacteria Bakteria docelowa	Combinations – Kombinacje K (n = 20)	 EM (n = 20)	 T (n = 20)
Lactobacillus spp.			
Frequency incidence Częstotliwość występowania	20/20 (100%)	20/20 (100%)	20/20 (100%)
CFU $\log_{10}$ ml^{-1} saliva – śliny	5.30a	5.55b	4.48c
Lactobacillus spp. (ability to produce H_2O_2) (zdolność do produkcji H_2O_2)			
Frequency incidence Częstotliwość występowania	17/20 (85%)	3/20 (15%)	13/20 (65%)
CFU $\log_{10}$ ml^{-1} saliva – śliny	4.70a	4.32b	3.90c

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

The identified *Lactobacillus* spp. isolates capable of produce hydrogen peroxide were subjected to PCR analysis (Phot. 1–3) for the presence of fragments of *recA*, *gyrB*, 16S rRNA genes.

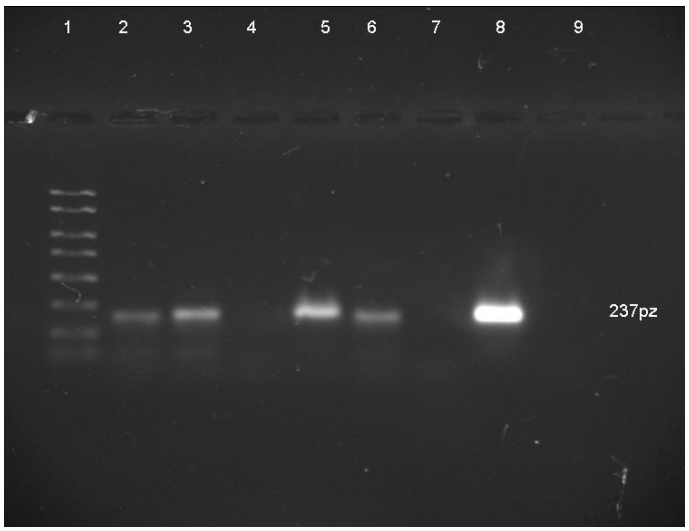
The performed PCR amplification allowed identification of the following species in individual combinations: K (11 strains of *Lactobacillus plantarum*, 6 strains of *L. brevis*); EM (3 strains of *L. plantarum*); T (7 strains of *L. buchneri*, 3 strains of *L. plantarum*, 3 strains of *L. brevis*).

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



Phot. 1. Separation of PCR products specific for *Lactobacillus plantarum* fragment gene *recA* (249 bp) in 1.5% agarose gel

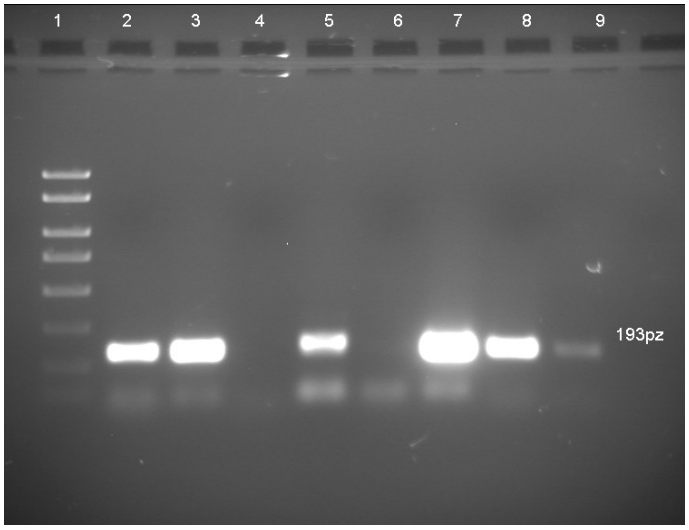
Fot. 1. Rozdział produktów PCR specyficznych dla fragmentu genu *recA* (249 pz) *Lactobacillus plantarum* w 1,5% żelu agarozowym



Phot. 2. Separation of PCR products specific for *Lactobacillus brevis* fragment gene *gyrB* (237 bp) in 1.5% agarose gel

Fot. 2. Rozdział produktów PCR specyficznych dla fragmentu genu *gyrB* (237 pz) *Lactobacillus brevis* w 1,5% żelu agarozowym

the composition of these preparations can produce 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].



Phot. 3. Separation of PCR products specific for *Lactobacillus buchneri* fragment gene 16S rRNA (193 bp) in 1.5% agarose gel

Fot. 3. Rozdział produktów PCR specyficznych dla fragmentu genu 16S rRNA (193 pz) *Lactobacillus buchneri* w 1,5% żelu agarozowym

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].

Among important factors inhibiting pathogen development and preventing their penetration into the gastrointestinal tract of animals is hydrogen peroxide produced by some strains of bacteria from *Lactobacillus* genus in quantities exceeding toxicity thresholds. In their experiments Adesokan et al. [2010] recorded high production of hydrogen peroxide by strains of *Lactobacillus brevis* and *Lactobacillus plantarum*. Among strains studied *in vitro* by these researchers, the *Leuconostoc mesenteroides* strain turned out to produce the smallest quantities of hy-

drogen peroxide. Different results were reported by Ogunbanawo [2005] who demonstrated the highest production of hydrogen peroxide *in vitro* by *Leuconostoc mesenteroides* strains. Zalán et al. [2005] observed that *Lactobacillus plantarum* produced large quantities of hydrogen peroxide but this strain failed to exhibit inhibitory properties in relation to *Escherichia coli*, although it inhibited growth of *Bacillus cereus* and *Listeria monocytogenes*. Results of many experiments demonstrated an inhibitory influence of hydrogen peroxide on such test strains of pathogens as: *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli* and *Listeria monocytogenes*. That is why it appears important to introduce probiotic preparation into feed containing in their composition lactic acid bacteria capable of manufacturing hydrogen peroxide [Patterson et al. 2008].

CONCLUSIONS

Revealed probiotics used varied significantly affect the abundance of *Lactobacillus* spp. strains. EM probiotic significantly restrict the development of *Lactobacillus* spp. are able to produce hydrogen peroxide. Toyo Cerin probiotic effect on reducing the number of *Lactobacillus* spp. strains in the mouths of dairy cows.

REFERENCES

Adesokan I.A., Ekanola Y.A., Okanlawon B.M. 2010. Influence of cultural conditions on hydrogen peroxide production by lactic acid bacteria isolated from some Nigerian traditional fermented foods. Afr. J. Microbiol. Res. 4, 1991–1996.

Barbosa T.M. 2000. The impact of antibiotics on resistance development and persistence. Drug Resist. Updat. 3, 303–311.

Casey P.G., Casey G.D., Gardiner G.E., Tangney M., Stanton C., Ross R.P., Hill C., Fitzgerald G.F. 2004. Isolation and characterisation of anti-*Salmonella* lactic acid bacteria from the porcine gastrointestinal tract. Lett. Appl. Microbiol. 39, 431–438.

Cao L.T., Wu J.Q., Xie F., Hu S.H., Mo Y. 2007. Efficacy of nisin in treatment of clinical mastitis in lactating dairy cows. J. Dairy Sci. 90, 3980–3985.

Ito A., Sato Y., Kudo S., Sato S., Nakajima H., Toba T. 2003. The screening of hydrogen peroxide producing lactic acid bacteria and their application to inactivating psychotrophic food borne pathogens. Curr. Microbiol. 47, 231–236.

Janik A., Koska M., Paluch U., Pieszka M., Barowicz T. 2006. Probiotics in piglet nutrition. Wiad. Zootech. 1, 3–9.

Khunajakr N., Wongwicharn A, Moonmangmee D., Tantipaiboonvut S. 2008. Screening and identification of lactic acid bacteria producing antimicrobial compounds from pig gastrointestinal tracts. KMITL Sci. Tech. J. 8, 8–17.

Klebanoff S.J., Hillier S.L., Eschenbach D.A., Waltersdorph A.M. 1991. Control of microbial flora of the vagina by H_2O_2-generating lactobacilli. J. Infect. Dis. 164, 94–100.

Ogunbanawo S.T. 2005. Functional properties of lactic acid bacteria isolated from ogi and fufu, two Nigeria fermented foods. Adv. Food Sci. 27, 14–21.

Osek J. 2003. Drug resistance of *Escherichia coli* strains of O157:H7 group isolated from humans and animals. Med. Weter. 59, 993–996.

Otero M.C., Morelli R., Nader-Macias M.E. 2006. Probiotic properties of bovine vaginal lactic acid bacteria to prevent mastitis. Lett. Appl. Microbiol. 43, 91–97.

Patterson J.L., Girerd P.H., Karjane N.W., Jefferson K.K. 2008. Effect of biofilm phenotype on resistance of *Gardnerella vaginalis* to hydrogen peroxide and lactic acid. Am. J. Obstet. Gynecol. 170, 171–177.

Pericone C.D., Overweg K., Hermans P.W., Weiser J.N. 2000. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of upper respiratory tract. Infect. Immunity. 68, 3990–3997.

Rekiel A. 2008. Effect of probiotic on the biochemical parameters in blood of fatteners. Med. Weter. 64, 110–112.

Rial D.R. 2000. The role of probiotic cultures in the control of gastrointestinal health. J. Nutr. 130, 396–420.

SAS 1999. SAS User's quide. Statistics version 7th ed., SAS Inst Inc Cary NC.

Sookkhee S., Chulasiri M., Prachyabrued A. 2001. Lactic acid bacteria from healthy oral cavity of Thai volunteers: inhibition of oral pathogens. J. Appl. Microbiol. 90, 172–79.

Zalán Z., Németh E., Baráth A., Halász A. 2005. Influence of growth medium on hydrogen peroxide and bacteriocin production of *Lactobacillus* strains. Food Technol. Biotechnol. 3, 219–225.

WPŁYW PROBIOTYKÓW NA LICZEBNOŚĆ PAŁECZEK KWASU MLEKOWEGO PRODUKUJĄCYCH NADTLENEK WODORU IZOLOWANYCH Z PYSKÓW KRÓW MLECZNYCH

Streszczenie. Doświadczenie prowadzono na 60 krowach mlecznych rasy holsztyńsko-fryzyjskiej (w wieku 3 lat o masie 590 kg) hodowanych w systemie uwięziowym. Zwierzęta podzielono na trzy grupy po 20 osobników w każdej. Grupę kontrolną (K) żywiono bez dodatku pobiotyków, grupę (EM) żywiono z dodatkiem EM probiotyku (dawka 150 ml $\cdot$ t^{-1} TMR), grupie (T) podawano w diecie probiotyk Toyo Cerin (dawka 0.2 kg $\cdot$ t^{-1} TMR). Od każdego zwierzęcia pobierano 2–10 cm^3 śliny, w której oznaczano: liczebność pałeczek bakterii mlekowych z rodzaju *Lactobacillus*, liczebność pałeczek zdolnych do produkcji nadtlenku wodoru. W celu precyzyjnej diagnostyki tych bakterii zastosowano testy biochemiczne API 50 CHL (BioMérieux) oraz pałeczki zdolne do produkcji nadtlenku wodoru poddano reakcji PCR. Występowanie pałeczek *Lactobacillus* spp. stwierdzono u wszystkich osobników w każdej kombinacji doświadczalnej. Pałeczki zdolne do tworzenia nadtlenku wodoru izolowano od 17 krów w grupie K, od 3 w grupie EM i 13 zwierząt w grupie T. Zastosowany EM probiotyk istotnie ograniczał rozwój szczepów *Lactobacillus* spp. zdolnych do produkcji nadtlenku wodoru.

Słowa kluczowe: *Lactobacillus* spp., nadtlenek wodoru, probiotyki

Accepted for print – Zaakceptowano do druku: 12.07.2013