COMPARATIVE EFFECTS OF DIETARY PHYTOBIOTIC (*MACLEAYA CORDATA* ALKALOID EXTRACT) AND PROBIOTIC (*PEDIOCOCCUS ACIDILACTICI* MA 18/5 M) PREPARATIONS AS SINGLE SUPPLEMENTS OR IN COMBINATION ON FERMENTATIVE PROCESSES IN THE BROILER CHICKENS CAECA

Jerzy Juskiewicz[1], Zenon Zdunczyk[1], Romas Gružauskas[2], Agila Daukšienė[2], Asta Racevičiūtė-Stupelienė[2], Žydrūnas Totilas[2]
[1]*Institute of Animal Reproduction and Food Research, Polish Academy of Sciences*
Tuwima 10, 10-747 Olsztyn, Poland
Phone: +48 89 523 4671; Fax : +48 89 524 0124 ; E-mail: j.juskiewicz@pan.olsztyn.pl
[2]*Veterinary Academy, Lithuanian University of Health Sciences, Tilžės 18, LT-47181, Kaunas, Lithuania*

Abstract. The aim of this 5-wk study was to characterize the physiological effects in the caeca of broilers fed diets containing two types of feed additives, as single supplements or in combination; an alkaloid preparation obtained from *Macleaya cordata* (Sangrovit, contaning mainly sanguinarine, 30 mg/kg) and a probiotic one (Bactocell, containing *Pediococcus acidilactici* MA 18/5 M, 1×10^6 CFU/g) were investigated. Caecal weight, pH of digesta, ammonia concentration as well as bacterial enzymes activity and short-chain fatty acids (SCFAs) concentration were assessed. Generally, the dietary treatments (n=10 birds, each) did not affect the caeca size, pH and ammonia concentration in the digesta. The enzymatic glycolytic activity of caecal microbiota was significantly increased in birds fed a diet containing probiotic ($P<0.05$ *v.* remaining groups in cases of β-galactosidase and β-glucosidase). The lowest glycolytic activity, proving antimicrobial properties, was found in chickens given the alkaloid preparation. As compared to the control unsupplemented diet, all preparations used in this study effectively reduced the activity of bacterial β-glucuronidase ($P<0.05$). The lowest concentration of total SCFAs was noted upon single Sangrovit treatment, and the highest one followed dietary combination of both preparations. In the latter case, the caecal concentration of butyric acid was markedly increased as compared to other groups. To sum up, it seems that the applied dietary combination of phytobiotic and probiotic preparations enables taking advantage of the physiological traits of both components (lower β-glucuronidase activity and higher, especially butyrate, SCFAs concentration in the broilers' caeca).

Keywords: broiler, caeca, bacterial enzyme activity, SCFAs, probiotic, sanguinarine.

LESALŲ, PAPILDYTŲ FITOBIOTIKAIS (*MACLEAYA CORDATA* ALKALOIDŲ EKSTRAKTU) IR/ARBA PROBIOTIKAIS (*PEDIOCOCCUS ACIDILACTICI* MA 18/5 M), POVEIKIS VIŠČIUKŲ BROILERIŲ AKLOSIOS ŽARNOS FERMENTACINIAMS PROCESAMS

Jerzy Juskiewicz[1], Zenon Zdunczyk[1], Romas Gružauskas[2], Agila Daukšienė[2], Asta Racevičiūtė-Stupelienė[2], Žydrūnas Totilas[2]
[1]*Gyvūnų reprodukcijos ir maisto tyrimų institutas, Lenkijos mokslų akademija*
Tuwima 10, 10-747 Olštinas
tel. +48 89 523 4671; faks. +48 89 524 0124; el. paštas: zez@pan.olsztyn.pl
[2]*Veterinarijos akademija, Lietuvos sveikatos mokslų universitetas*
Tilžės g. 18, LT-47181, Kaunas

Santrauka. Šiuo 5 savaičių trukmės bandymu norėta nustatyti dviejų skirtingų lesalų priedų ir jų derinio – alkaloidų, gautų iš *Macleaya cordata* („Sangrovit", veiklioji medžiaga sangvinarinas, 30 mg/kg) ir probiotikų („Bactocell", sudarytų iš *Pediococcus acidilactici* MA 18/5 M, 1×10^6 KSV/g) poveikį viščiukų broilerių aklosios žarnos fiziologiniams procesams. Tirti šie rodikliai: aklosios žarnos svoris, chimuso pH, amoniako koncentracija, bakterinių enzimų aktyvumas ir trumpų grandinių riebalų rūgščių (TGRR) kiekis. Nustatyta, kad lesalų priedai nedarė įtakos aklosios žarnos svoriui, chimuso pH ir amoniako koncentracijai. Viščiukų, gavusių lesalus su probiotikų priedu, palyginti su kontroline ir kitomis tiriamosiomis grupėmis aklosios žarnos mikrofloros enzimatinis glikolitinis aktyvumas (beta galaktozidazės ir beta gliukozidazės; $p<0,05$) padidėjo. Mažiausias glikolitinis aktyvumas nustatytas viščiukų, lesintų alkaloidinio preparato priedu. Palyginti su kontroline grupe fitobiotinis ir probiotinis preparatai ženkliai sumažino bakterinio enzimo beta gliukuronidazės aktyvumą ($p<0,05$). Mažiausias bendras TGRR kiekis buvo dėl „Sangrovit" įtakos, o didžiausias – veikiant fitobiotinio ir probiotinio preparatų deriniui. Be to, dėl abiejų preparatų derinio lesaluose viščiukų aklosios žarnos chimuse ženkliai padaugėjo sviesto rūgšties palyginti su kontroline ir kitomis tiriamosiomis grupėmis. Apibendrinant galima teigti, kad fitobiotikų ir probiotikų derinys turi teigiamos įtakos aklosios žarnos fiziologiniams procesams (sumažėja beta gliukuronidazės aktyvumas ir padidėja bendras TGRR kiekis, ypač sviesto rūgšties).

Raktažodžiai: broileriai, akloji žarna, bakterinių enzimų aktyvumas, TGRR, probiotikas, sangvinarinas.

Introduction. Recently, health status of the gut, especially its lower part (Mikulski et al., 2011, Zdunczyk et al., 2010a), has become an object of concern along with the productivity of birds. In this regard, a combinatory use of selected feed additives has been investigated as a better way to control and improve the intestinal environment (Juskiewicz et al., 2009; Semaskaite et al., 2009). In broilers, as in almost all birds, the caeca are the main site of the bacterial fermentative processes in the gastrointestinal tract (Józefiak et al., 2004). This fermentation converts undigested intestinal contents into biomass, short-chain fatty acids and gases. The pattern of bacterial enzymatic activity and, then, this of SCFAs are paramount factors of nutritional importance, which influence (beneficially or adversely) the caecal mucosa as well as lipid and carbohydrate metabolism (Jurgonski et al., 2008). The idea presented in this study, concerning a dietary combination of phytobiotics and living beneficial bacteria, may be considered as a new approach in the feeding systems. So far, the majority of literary sources deal with the animal feeding with simultaneous addition of a probiotic with a prebiotic, which is known as a symbiotic supplement (Juskiewicz et al., 2007; Semaskaite et al., 2006). Sanguinarine and chelerythrine are the main active components of preparation Sangrovit, an extract from *Macleaya cordata* (Willd.) R.Br. (*Papaveraceae*); these two quanternary benzo[*c*]phenanthridine alkaloids (QBAs) are known to have antimicrobial, anti-inflammatory and immune-modulatory effects in farm animals (Jankowski et al., 2009; Juskiewicz et al., 2011). Moreover, in the gut, sanguinarine exerts inhibitory activity of aromatic amino acid decarboxylase, thus suppresses the adverse effects of some fungi and bacteria (e.g. lowered production of biogenic amines and toxins) (Mellor, 2001). It has been reported that *Pediococcus acidilactici* exhibited a strong capacity for surviving acidic and bile salt conditions (Erkkila and Petaja, 2000), thus taking into account that most of the probiotic strains colonize the gut only temporarily, we expect that a simultaneous addition of dietary Sangrovit and preparation Bactocell containing *Pediococcus acidilactici* may better act together than separately to the broilers' caecal environment. Therefore, in the present study the following hypothesis was advanced: the concomitant presence of phytobiotic with probiotic bacteria strain in a broilers' diet may provide additional effects to the caecal metabolism, what enables taking advantage of the physiological traits of both components.

Material and Methods. The procedures related to birds care used in this experiment followed the International Guiding Principles for Biomedical Research Involving Animals as Issues by the Council for the International Organizations of Medical Sciences and EU Directive 86/609/EEC and EC recommendations 2007/526 EC „Using and keeping of animals for experimental and other purposes". The physiological study was conducted on 40 one-day-old Cobb 500 broiler chickens allocated to four groups, each of 10 chickens. The birds were kept individually in metabolic cages and had free access to compound feed and tap water. During the first week of the experiment, the chicks were maintained on 24 hours light schedule, and an 18 hours light schedule was used thereafter.

The birds were fed for 5 weeks (a starter and grower diets from day 1 to 8 and 9 to 35, respectively) *ad libitum* with a crumbled wheat-corn-soybean meal based diet (control group, C) supplemented with an alkaloid preparation (A group, Sangrovit, 30 mg/kg of feed, Phytobiotics GmbH, Etville, Germany), with a probiotic preparation (B group, Bactocell, Lalleman Inc., Blagnac, France; 1×10^6 CFU/g), or with a dietary combination of both additives (group AB). A basal diet was formulated to meet the nutrient and energy requirement for broiler chickens (NRC, 1994) and is presented in Table 1. The content of pure sanguinarine in the experimental diet was analytically estimated at 0.36 mg/kg. Sanguinarine was determined in a diet by reverse-phase HPLC using a C_{18} column with phosphate buffer-acetonitrile-triethyloamine (65:34:1, v/v) as a mobile phase and with 330 nm excitation and 570 nm emission detection (Jankowski et al., 2009).

Table 1. **Nutritional value of the basal diet**, %

Nutrient	Starter 1–8 d	Grower 9–35 d
Crude protein	22.80	22.30
Crude fat	4.90	6.50
Crude fiber	2.60	2.50
Crude ash	3.40	3.00
Lysine	1.33	1.27
Methionine	0.70	0.65
Methionine/Cysteine	1.06	1.00
Tryptophan	0.27	0.27
Threonine	0.89	0.84
Linoleic acid	1.47	1.85
Ca	1.02	0.97
P (total)	0.74	0.73
P (available)	0.49	0.46
Na	0.19	0.17
Cl	0.25	0.20
K	0.82	0.82
Mg	0.18	0.18
Metabolizable energy[1] (kcal/kg)	2949	3076

[1]Calculated using formula: ME (kcal/kg) = 10[(3.5×CP) + (8.5×CF) + (3.5×NFE)]; where ME, metabolizable energy; CP, crude protein (%); CF, crude fat (%); NFE, nitrogen free extract (%)

Diet samples were analyzed in duplicate for crude protein (CP), fat, crude fiber (CF), and ash using Association of Official Analytic Chemists (2005) methods 976.05, 920.39, 978.10, and 942.05, respectively. For chemical analysis, the samples were ground to pass through a 0.5 mm sieve. The calcium content was determined using a Perkin-Elmer 1100B atomic absorption spectrophotometer. Phosphorus content was determined using the AOAC (2005) method 965.17.

At the end of the trial, the broiler chickens were killed by cervical dislocation according to the recommendations for euthanasia of experimental animals. The caeca with contents were taken from each bird, and as soon as possible after euthanasia (ca. 30 minutes), caecal pH was measured using a microelectrode and pH/ION meter (model 301, Hanna Instruments, Vila do Conde, Portugal). Samples of caecal contents were used for immediate analysis: ammonia, dry matter, short-chain fatty acids (SCFAs), while the rest of digesta was transferred to tubes and stored at -70°C. The caecal wall was flushed clean with ice-cold saline water, blotted on filter paper, and weighed (caecal wall weight). Dry matter of caecal digesta was determined at 105ºC. In fresh caecal digesta samples, ammonia was extracted and trapped in a solution of boric acid in Conway dishes and was determined by direct titration with sulfuric acid. The bacterial glycolytic activity in the caecal digesta was measured by the rate of *ρ*- or *o*- nitrophenol release from their nitrophenylglucosides according to the modified method of Djouzi and Andrieux described by Juskiewicz et al. (2002). The following substrates were used: *ρ*-nitrophenyl-*α*-D-glucopyranoside (for *α*-glucosidase), and *ρ*-nitrophenyl-*β*-D-glucopyranoside (for *β*-glucosidase), *ρ*-nitrophenyl-*α*-D-galactopyranoside (*α*-galactosidase), *o*-nitrophenyl-*β*-D-galactopyranoside (*β*-galactosidase), and *ρ*-nitrophenyl-*β*-D-glucuronide (for *β*-glucuronidase). The caecal content was diluted (100 g/L) in 100 mM phosphate buffer (pH 7.0). The suspension was centrifuged for 15 minutes at 7,211 × g at room temperature, and enzymes were assayed on the supernatant. The reaction mixture contained 0.3 mL of a substrate solution (5 mM) and 0.2 mL of a dilution of the caecal sample. Incubation was carried out at 37ºC and *ρ*-nitrophenol was quantified at 400 nm and at 420 nm (*o*-nitrophenol concentration) after the addition of 2.5 mL of 0.25 M cold sodium carbonate. The enzymatic activity (*α*- and *β*-glucosidase, *α*- and *β*-galactosidase, and *β*-glucuronidase) was expressed as µmol product formed per hour per g of digesta. Caecal digesta samples were subjected to the SCFAs analysis using gas chromatography (Shimadzu GC-2010; Shimadzu, Kyoto, Japan). The samples (0.2g) were mixed with 0.2 mL formic acid, diluted with deionized water and centrifuged at 7,211 × g for 10 min. The supernatant was loaded onto a capillary column (SGE BP21, 30 m × 0.53 mm) using an on-column injector. The initial oven temperature was 85ºC and was raised to180ºC by 8ºC /min and held there for 3 minutes. The temperatures of flame ionization detector and the injection port were 180 and 85ºC, respectively. The sample volume for GC analysis was 1 µL.

The results of the experiment were analyzed using the 1-way ANOVA test, and significant differences between groups were determined by Duncan's multiple range test. Statistica 8.0. for Windows™ software was used. Data in tables are given as means ± SEM. Differences were considered significant at $P<0.05$.

Results. All dietary treatments applied in this study did not affect the final body weight of broilers (data not shown). The bulk, pH value and dry matter concentration of digesta as well as relative tissue mass of the caeca were similar in all groups ($P>0.05$; Table 2). Caecal ammonia concentration ranged from 0.50-0.51 mg/g in the A and AB groups (phytobiotic and phytobiotic plus probiotic dietary treatments, respectively) to 0.56-0.57 mg/g of digesta in groups C and B (control and probiotic treatments, respectively), but did not differ significantly among them ($P=0.689$).

Table 2. **Caecal indices of broiler chickens**

	Experimental groups				*P* value
	C	A	B	AB	
Caecal tissue, g/kg BW	2.36±0.13	2.39±0.20	2.42±0.13	2.41±0.16	0.823
Caecal digesta, g/kg BW	4.22±0.43	4.85±0.63	4.34±0.43	4.66±0.79	0.352
Dry matter, %	16.2±0.46	16.7±0.98	16.5±0.46	17.1±0.31	0.141
Ammonia, mg/g	0.56±0.01	0.50±0.01	0.57±0.01	0.51±0.01	0.689
pH of digesta	6.46±0.22	6.14±0.36	6.89±0.22	6.48±0.28	0.437

C – control, without supplements, A – supplemented with phytobiotic, B – supplemented with probiotic, AB – supplemented with phytobiotic and probiotic

Table 3. **Activity of microbial enzymes in the caecal digesta, µmol/h/g**

Enzymes, µmol/h/g	Experimental groups				*P* value
	C	A	B	AB	
α-glucosidase	36.3 ± 2.01^{b}	24.5 ± 0.98^{c}	44.2 ± 2.42^{a}	42.7 ± 3.46^{ab}	>0.001
β-glucosidase	18.1 ± 0.83^{b}	7.67 ± 0.63^{d}	22.5 ± 1.06^{a}	12.6 ± 1.27^{c}	>0.001
α-galactosidase	51.6 ± 4.52^{a}	24.6 ± 2.69^{b}	55.6 ± 3.96^{a}	46.0 ± 2.08^{a}	>0.001
β-galactosidase	29.9 ± 5.69^{b}	15.0 ± 1.06^{c}	48.4 ± 5.20^{a}	29.3 ± 3.71^{b}	>0.001
β-glucuronidase	65.6 ± 5.43^{a}	23.3 ± 1.49^{c}	51.8 ± 2.98^{b}	41.7 ± 3.98^{b}	>0.001

Values with the different letters differ significantly; abcd – $P<0.05$; C – control, without supplements, A – supplemented with phytobiotic, B – supplemented with probiotic, AB – supplemented with phytobiotic and probiotic

Table 4. **Short chain fatty acids (SCFAs) concentration in the caecal content**

	Experimental groups				*P* value
	C	A	B	AB	
SCFAs, μmol/g					
acetate	79.7±2.80^{a}	69.8±2.34^{b}	83.2±1.69^{a}	80.5±3.06^{a}	0.004
propionate	13.4±0.85^{a}	11.0±0.86^{b}	11.3±0.49^{b}	10.5±0.65^{b}	0.039
iso-butyrate	1.55±0.13^{a}	0.81±0.04^{b}	0.91±0.12^{b}	0.80±0.05^{b}	>0.001
butyrate	21.4±1.06^{c}	24.0±0.71^{c}	28.1±1.12^{b}	34.4±2.15^{a}	>0.001
iso-valerate	1.99±0.30^{a}	1.08±0.11^{b}	1.48±0.26^{ab}	1.24±0.12^{b}	0.025
valerate	2.24±0.23	2.28±0.23	2.27±0.24	2.71±0.16	0.386
total	120±3.58^{b}	109±2.83^{c}	127±2.03^{ab}	130.5±5.26^{a}	0.001
Profile of SCFA, %					
C2	66.2±0.84^{a}	64.0±0.95^{ab}	65.4±1.05^{a}	61.9±0.58^{b}	0.008
C3	11.1±0.56^{a}	10.1±0.69^{ab}	8.89±0.35^{bc}	8.10±0.41^{c}	0.012
C4	17.9±0.95^{a}	22.2±0.84^{b}	22.1±0.74^{b}	26.3±0.94^{a}	>0.001

Values with the different letters differ significantly; abc – P<0.05; C – control, without supplements, A – supplemented with phytobiotic, B – supplemented with probiotic, AB – supplemented with phytobiotic and probiotic

The addition of selected feed additives significantly affected the glycolytic activity of microflora in the caecal digesta (Table 3). The highest activity of bacterial α- and β-glucosidases as well as α- and β-galactosidases were noted in birds fed a diet containing probiotic bacteria (P<0.05 *v*. remaining groups in cases of β-galactosidase and β-glucosidase). The lowest glycolytic (including all the aforementioned enzymes) and β-glucuronidase activity was found in chickens fed a diet with alkaloid preparation (P<0.05 *v*. all treatments). Furthermore, the highest activity of bacterial β-glucuronidase was in the broilers from the control group (P<0.05).

The caecal concentration of total short-chain fatty acids in the experimental groups was as follows: $AB^{a}>B^{ab}>C^{b}>A^{c}$. The low concentration of total SCFAs following the single phytobiotic dietary addition was mainly due to a significant decrease in acetic acid concentration as compared to all other groups (P<0.05). The highest caecal concentrations of propionate, iso-butyrate (P<0.05 *v*. other groups) and iso-valerate (P<0.05 *v*. A and AB groups) were detected after the control (not supplemented) treatment. The combinatory addition of both supplements (AB group) was associated with the highest butyric acid concentration in the caecal digesta. The analysis of SCFAs C2:C3:C4 profile points to a lower ratio of acetic and propionic acids under the influence of the AB diet feeding (P<0.058 *v*. other groups as well as P<0.05 *v*. C and A groups, respectively). The phytobiotic and probiotic treatments as single supplements were characterized by a lower proportion of butyrate in comparison to the control- and AB-treated broilers.

Discussion. The growth of all chickens in this study proceeded normally. Available data regarding Sangrovit use in poultry nutrition are still scarce and inconsistent, but to our knowledge there have been no reports that sanguinarine-treated birds (up to 50 mg/kg of a diet) grew slower than their untreated counterparts (Juskiewicz et al., 2011). The idea behind the use of plant preparations containing QBAs is to maintain good health status and increase performance by enhanced feed consumption (improved appetite) and digestion (Mellor, 2001). The effect of sanguinarine in internal organs is of less importance as it is slowly absorbed in the gastrointestinal tract, and the main activity of this alkaloid is associated with the gut (Jankowski et al., 2009; Zdunczyk et al., 2010b). Similarly, as the improvement of gut health is the main target of probiotic addition, it justifies our research approach in this study to focus on the caecal physiological response. Contrary to expectations, neither the phytobiotic nor probiotic supplements, as single or combination treatments, affected ammonia concentration in the caeca. One of the physiological properties of alkaloid sanguinarine is inhibition of the intestinal aromatic amino acid decarboxylase activity, thus this class of amino acids could be better utilized by animals (Vieira et al., 2008). Better protein utilization is commonly associated with decreased passage of unabsorbed peptides and amino acids into lower parts of the gut, leading to diminished bacterial ammonia as well as putrefactive SCFAs formation (Juskiewicz et al., 2005). Indeed, the dietary addition of phytobiotic preparation Sangrovit reduced caecal concentration of branched-chain fatty acids (BCFA, iso-butyric and iso-valeric acids) as compared to the control group (P<0.05 *v*. A and AB treatments). On the other hand, a similar tendency towards lower BCFA content was observed upon the B treatment (probiotic Bactocell addition, P<0.05 *v*. C in the case C4i concentration, and insignificant decrease in the case of C5i concentration in the caecal digesta). In our previous work on small laboratory rodents, the supplementation of a diet with prebiotic strain *Pediococcus acidilactici* did not affect the caecal concentration of BCFA or valerate, either as it was applied as single dosage or when it was combined with fructooligosaccharides or polysaccharidases (Juskiewicz et al., 2007). In this cited study on rats, the addition of probiotic *P. acidilactici* caused a more beneficial composition (profile) of SCFAs (especially, as single supplement, and to lesser extent when combined with FOS), with a higher content of

propionic acid. Such an effect was not observed in the present study on broiler chickens. It is worth of notice a higher concentration of butyric acid following the combinatory use of two tested feed additives, alkaloid phytobiotic and probiotic bacteria. The applied single treatments or the control diet did not cause this effect. Butyric acid, in humans and animals including poultry, is claimed to improve gut integrity (when present in the blood or in the intestinal tract increases the production of specific peptides stimulating normal cell proliferation) and the functioning of the immune system (Leeson et al., 2005).

It should be assumed that in the present experiment the dietary sanguinarine preparation added at a dose of 30 mg/kg diet suppressed the fermentation processes in the caeca as indicated by decreased glycolytic enzymes activities and production of SCFAs. Those effects were not observed when concomitant addition of phytobiotic and probiotic was experimentally applied. Considering the fact that the dietary AB treatment diminished caecal β-glucuronidase and β-glucosidase, but not α-glucosidase, α-galactosidase, and β-galactosidase activities in comparison to the control group, a conclusion could be drawn that the *Macleaya cordata* extract added to a diet at the level of 30 mg/kg together with probiotic strain *Pediococcus acidilactici* exerted selective modulation effect on the caecal microbiota. β-galactosidase, α-galactosidase and α-glucosidase activities can improve the fermentation of lactose, raffinose family oligosaccharides and resistant starch leading to SCFA and lactic acids which are a source of energy for the tissues. On the other hand, the β-glucuronidase and β-glucosidase activity levels are often used as markers of pathogenic microflora activity leading to unwanted metabolic changes (Juskiewicz et al., 2006). The aforementioned modulation effect of the AB dietary treatment on caecal microflora was also supported by the enhanced total SCFAs ($P<0.05$ *v*. C and B groups).

Conclusion. The applied dietary combination of alkaloid preparation from *Macleaya cordata* and probiotic preparation containing *Pediococcus acidilactici* provided an additional (as compared to single treatments not only to control group) physiological effect in the gut metabolism as indicated by lower bacterial β-glucuronidase and β-glucosidase activities and higher, especially butyrate, SCFAs concentration in the broilers' caeca.

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