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THE EFFECT OF A PROBIOTIC PREPARATION CONTAINING *BACILLUS SUBTILIS* PB6 IN THE DIET OF CHICKENS ON REDOX AND BIOCHEMICAL PARAMETERS IN THEIR BLOOD*

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Abstract

The aim of the study was to select a dosage and time of administration of a probiotic preparation containing live cultures of *Bacillus subtilis* and enriched with choline to obtain the most beneficial effect on the antioxidant and biochemical status of the blood of chickens and to improve their growth performance. A total of 980 one-day-old Ross 308 chickens (7 replications of 20 individuals each) reared until their 42nd day of life were used in the experiment. The chickens were divided into seven groups of 140 each. The control group did not receive any additives. The T1 groups received a probiotic in the amount of 0.05 g/L (T1-0.05), 0.1 g/l (T1-0.1) or 0.25 g/l (T1-0.25) throughout the rearing period, while the T2 groups received the same doses of the probiotic, but only during days 1–7, 15–21 and 29–35 of rearing. Administration of a preparation containing *Bacillus subtilis* bacteria was shown to increase the level of ferric reducing ability of plasma (FRAP), vitamin C, and uric acid (UA), while reducing the level of peroxides (LOOH), malondialdehyde (MDA), non-esterified fatty acids (NEFA), the share of low-density fractions of cholesterol (LDL), and activity of alanine aminotransferase (ALT), asparagine aminotransferase (AST), γ-glutamyltransferase (GGT) and creatinine kinase (CK). An increase in the high-density fractions of cholesterol (HDL) and a decrease in lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were noted as well. The results of the study indicate that 0.25 g/l of the probiotic, administered continuously (T1), clearly has the most beneficial effect in terms of enhancing antioxidant potential and reducing the level of stress indicators, without disturbing overall metabolism in the body. During the 42 days of rearing each chicken received 33.3 CFUx10^{11} *Bacillus subtilis* from the probiotic preparation. The body weight gain of chickens from T1-0.1, T1-0.2 and T2-0.25 groups was higher (P≤0.027) and more favourable compared to G–C group.

Key words: probiotic, *Bacillus subtilis*, choline, broilers, redox reaction, biochemical parameters

The continual search for new solutions to improve the health and growth performance of poultry (Ognik and Krauze, 2012; Khan and Naz, 2013; Abudabos et

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al., 2017; Sobczak and Kozłowski, 2015; Jankowski et al., 2016, 2017; Ognik et al., 2017) has led to the use of probiotics, which may be helpful in stimulating digestion eliminating pathogens (Yeoman et al., 2012), and reducing mortality caused by disease (Park et al., 2014; Park and Kim, 2014). Numerous reports indicate the benefits of the use of various probiotic species and strains in poultry rearing (Abudabos et al., 2017; Al-Sagan and Abudabos, 2017; Fathi et al., 2018; Zarei et al., 2018). However, there have been few attempts to select the optimum dose and time of administration of these additives in order to obtain the most beneficial impact on both the health of birds and their performance. This seems to be an important question due to the very strong metabolic activity of probiotic microorganisms, which can determine the physiological and defence responses of the colonized organism. According to scientific reports, *Bacillus subtilis* bacteria can improve the development and growth of birds, their utilization of nutrients, and the antioxidant and biochemical status of the blood (Abudabos et al., 2017), favourably alter the morphometry and microbiome of the gut (Yeoman et al., 2012; Park and Kim, 2015; Chand et al., 2016; Oh et al., 2017), and enhance immunity (Lee et al., 2013), without disturbing nutrition processes in chickens (Zhang and Kim, 2014; Nguyen et al., 2015; Mahmoud et al., 2017). Choline, on the other hand, performs important metabolic functions, is responsible for the synthesis of cell membrane phospholipids, is a precursor of acetylcholine, participates in lipid metabolism in the liver, and protects it against steatosis. Choline, like betaine and methionine, provides methyl groups for biochemical reactions in the body. In a two-step enzymatic reaction in the hepatocyte mitochondria, choline is oxidized to betaine, which can be a methyl group donor for the methylation reaction. This betaine is transformed into methionine, which as a methyl group donor is crucial for maintaining other metabolic reactions conditioning homeostasis. It contributes to DNA protection and methylation of proteins, and it is essential for protein synthesis and detoxification. According to Zeisel and da Costa (2009), much of the choline in the feed ration is used to produce methionine. A deficiency of methyl groups in the diet contributes to the occurrence of glutathione deficiencies. It also prevents detoxification of homocysteine, which leads to cell membrane damage due to oxidative stress. Appropriate choline supplementation prevents perosis and chondrodystrophy (Farina et al., 2017) and improves growth performance in poultry (Igwe et al., 2015). However, opinions about the effectiveness of choline supplementation to improve performance results are divided. Some authors claim that the addition of choline to the basal feed increases weight gain and reduces feed consumption (Igwe et al., 2015). According to Pompeu et al. (2011), choline only improves FCR, while Swain et al. (2000) argue that such supplementation has no benefits in the form of improved growth performance.

The modification of redox status caused by increased generation of reactive oxygen species is associated with a change in the activity of antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, or catalase, and in the level of non-enzymatic antioxidants, including glutathione, vitamin C, albumin, bilirubin, uric acid and tocopherol, as well as harmful metabolites, such as malondialdehyde or lipid peroxides (Douglas et al., 2011). Particularly susceptible to the toxic effects of free radicals are polyunsaturated fatty acids of cell membrane phospholipids, which

undergo peroxidation. This contributes to a loss of cell membrane integration and permeability, resulting in the release of cellular lysosomal enzymes and changes in the affinity of receptors and antigenic determinants. Choline deficiency in phospholipids also causes cell membrane fragility, thereby impeding transport of triacylglycerols and cholesterol, which leads to accumulation of cholesterol in the liver. Lipid peroxidation end products and by-products (e.g. malondialdehyde) interfere with protein synthesis by damaging vascular permeability and disrupting the body's immune defences. Oxidative damage to proteins, both structural and enzymatic, causes changes in the structure and activity of these molecules (Douglas et al., 2011).

We postulated that by using a probiotic in chicken diets, the antioxidant system of the birds can be stimulated without inducing oxidative stress or disturbing metabolic processes.

The aim of the experiment was to select the dose and time of administration of a preparation containing live cultures of *Bacillus subtilis*, enriched with choline, which would most favourably influence redox status without adversely affecting the blood metabolic profile or growth performance of the chickens.

Material and methods

Probiotic

The subject of the study was a commercial product containing live *Bacillus subtilis* PB6 and choline (Industries Inc., USA). The product dissolved in one litre of water contains *Bacillus subtilis* PB6 in the amount of $2.0x10^9$ CFU and 1500 mg of choline.

Animals

The material for the study consisted of day-old Ross 308 male chickens raised until the age of 42 days. The experimental procedure was approved by the Second Local Ethics Committee for Experiments with Animals in Lublin (approval no. 38/2018). The birds were kept in pens on straw litter and reared in standard conditions in a building with regulated temperature and humidity. They had permanent access to drinking water and received *ad libitum* complete compound feeds appropriate for the rearing period in accordance with feeding standards for poultry (Nutrient Requirements for Poultry, 2005) (Table 1).

The experimental design for administration of the probiotic preparation is shown in Table 2. The experiment was carried out on 980 chickens assigned to seven experimental groups of 140 birds each (7 replications of 20 individuals each). The control group (G–C) did not receive the probiotic. Groups T1-0.05, T1-0.1 and T1-0.25 received the probiotic in their drinking water in the amount of 0.05 g/l for group T1-0.05, 0.1 g/l for group T1-0.1, and 0.25 g/l for group T1-0.25 on days 1–42 of life. The birds in groups T2-0.05, T2-0.5 and T2-0.25 received the probiotic in the same amounts, but only on days 1–7, 15–21 and 29–35 of life.

Table 1. Composition of mixtures for chicken (g/kg)

Components	Starter 1–3 weeks	Grower 4–5 weeks	Finisher 6 week
Wheat	452.8	367.63	330.70
Maize	150.0	250.0	300.0
Soybean meal, 46% protein	272.21	227.90	178.09
Rapeseed meal, 37% protein	20.0	40.0	60.0
Soybean oil	20.0	40.0	60.0
DDGS[1], 26% protein	40.07	43.58	46.87
Monocalcium phosphate	11.03	5.42	2.05
Coarse-grained ground limestone	–	10.93	8.52
Fine-grained ground limestone	16.07	–	–
NaCl	3.63	3.23	2.83
DL-methionine 99%	3.61	2.40	2.00
L-lysine HCl	4.27	2.97	3.12
L-threonine 99%	1.31	0.94	0.82
Premix[2]	5	5	5
Calculated nutrient composition of mixture (g/kg)			
Crude protein	210.0	198.5	187.5
Crude fibre	27.2	29.8	32.2
Crude fat	65.9	74.5	81.4
Lysine	13.5	11.7	10.9
Methionine	6.7	5.5	5.0
Methionine + Cysteine	10.1	8.8	8.3
Tryptophan	2.5	2.3	2.1
Arginine	13.1	12.1	11.1
Total calcium	9.8	7.3	6.0
Available phosphorus	3.9	2.8	2.1
Sodium	1.6	1.5	1.4
Metabolizable energy (kcal/kg)	3070	3140	3190

[1]DDGS – maize distillers dried grains with solubles.

[2]Premix – **1–3 weeks**: retinol – 1034 mg/kg; cholecalciferol – 25 mg/kg; tocopherol – 15 g/kg; menadione – 0.8 g/kg; thiamine – 0.6 g/kg; riboflavin – 1.6 g/kg; pyridoxine – 1 g/kg; cobalamin – 3.2 g/kg; folic acid – 0.4 g/kg; biotin – 40 mg/kg; nicotinic amide – 12 g/kg; calcium pantothenicum – 3.6 g/kg; choline – 360 g/kg; manganese – 20 g/kg; zinc – 16 g/kg; iron – 16 g/kg; copper – 1.6 g/kg; iodine – 0.2 g/kg; selenium – 30 mg/kg; coccidiostat – salinomycin; **4–5 weeks**: retinol – 827 mg/kg; cholecalciferol – 25 mg/kg; tocopherol – 10 g/kg; menadione – 0.6 g/kg; thiamine – 0.4 g/kg; riboflavin – 1.2 g/kg; pyridoxine – 0.8 g/kg; cobalamin – 3.2 g/kg; folic acid – 0.35 g/kg; biotin – 10 mg/kg; nicotinic amide – 12 g/kg; calcium pantothenicum – 3.6 g/kg; choline – 320 g/kg; manganese – 20 g/kg; zinc – 16 g/kg; iron – 16 g/kg; copper – 1.6 g/kg; iodine – 0.2 g/kg; selenium – 30 mg/kg; coccidiostat – salinomycin; **6 week**: retinol – 827 mg/kg; cholecalciferol – 25 mg/kg; tocopherol – 10 g/kg; menadione – 0.4 g/kg; thiamine – 0.4 g/kg; riboflavin – 1 g/kg; pyridoxine – 0.6 g/kg; cobalamin – 2.2 g/kg; folic acid – 0.3 g/kg; biotin – 10 mg/kg; nicotinic amide – 7 g/kg; calcium pantothenicum – 3.6 g/kg; choline – 320 g/kg; manganese – 20 g/kg; zinc – 16 g/kg; iron – 16 g/kg; copper – 1.6 g/kg; iodine – 0.2 g/kg; selenium – 30 mg/kg.

Table 2. The experimental scheme applied dose of probiotic preparation containing *Bacillus subtilis* PB6 for chickens

Item	Treatment						
	C[2]	$T1_{0.05}$	$T1_{0.1}$	$T1_{0.25}$	$T2_{0.05}$	$T2_{0.1}$	$T2_{0.25}$
Cycle administration of probiotic preparation[1]	0	6 × 7	6 × 7	6 × 7	3 × 7	3 × 7	3 × 7
Total intake of probiotic preparation, g/bird	0	0.165	0.331	0.827	0.059	0.118	0.297
Intake of choline 1–42 days, mg/bird[3]	7.56	7.916	8.53	10.15	7.47	7.89	8.54
Total intake *Bacillus subtilis* PB6[4], CFU × 10^{11}/bird	0	6.6	13.3	33.3	2.9	5.7	14.3

[1]6 × 7 intake in 1–42 days of chicken life or 3 × 7 intake on 1–7, 15–21 and 29–35 days of chicken life.

[2]In C group only in fodder intake on 1–42 days of life, in other groups total choline intake in the fodder and probiotic preparation in their drinking water.

[3]Choline from preparation and fodder.

[4]C group did not receive the probiotic preparation on 1–42 days of life, in other groups total *Bacillus subtilis* intake only in the probiotic preparation in their drinking water.

Laboratory analysis

On day 42 of rearing, blood samples were collected for analysis from the wing vein of 21 chickens from each group (three each from each replicate group), after two-hour fasting. The blood samples were cooled and analysed within 4 hours from collection. They were centrifuged at 3000 g for 10 minutes to collect serum for further analysis. Kits developed by Cormay® were used to determine biochemical parameters in the plasma: uric acid (UA), bilirubin (BIL), creatinine (CREAT), cholesterol (TC) and its high-density (HDL) and low-density (LDL) fractions, and triacylglycerol (TAG). The activity of the following enzymes was determined in the plasma: alanine aminotransferase (ALT; EC 2.6.1.2), asparagine aminotransferase (AST; EC 2.6.1.1), creatinine kinase (CK; EC 2.3.7.2), lactate dehydrogenase (LDH; EC 1.1.1.27), γ-glutamyltransferase (GGT; EC 2.3.2.2), alkaline phosphatase (ALP; EC 3.1.3.1), acidic phosphatase (AC; EC 3.1.3.2) and 3-hydroxybutyrate dehydrogenase (HBDH; EC 1.1.1.30). The level of nonesterified fatty acids (NEFA) was determined using reagents by Randox®. The activity of antioxidant enzymes in the plasma was analysed using spectrophotometric assays. To evaluate the activity of superoxide dismutase (SOD, EC 1.15.1.1), the adrenaline method was used with a modification of the wavelength to 320 nm (Misra and Fridovich, 1972). Catalase (CAT, EC 1.11.1.6) activity was analysed according to Aebi (1984), concentrations of ascorbic acid (VIT. C) were determined according to Omaye et al. (1979), and the glutathione level (GSH + GSSG) according to Akerboom and Sies (1981). The ferric reducing ability of plasma (FRAP), which represents total antioxidant capacity, was determined according to Benzie and Strain (1996). The level of peroxides (LOOH) was determined according to Gay and Gębicki (2002) and malondialdehyde (MDA) according to Salih et al. (1987).

Statistical analysis

The model assumptions of normality of variance were verified by the Shapiro–Wilk test, and homogeneity of variance was analysed using Levene's test. The results were analysed by one-way ANOVA. Planned contrast analysis was used to compare

the control group (G–C) with all other experimental groups. In addition, Dunnett's two-tailed post-hoc test was used to compare the control group (G–C) with each experimental group separately. In a model without the G–C group, the following effects were examined by two-way ANOVA: D – dose effect, T – time effect, and D×T – interaction between dose and time. In the case of a significant interaction effect in the results, the Newman–Keuls test was used to evaluate the differences between the factors. The GLM procedure in Statistica 13.0 PL software (StatSoft Corp®) was used for the statistical analysis. Treatment effects were considered significant at $P \leq 0.05$. All data were expressed as mean values with pooled standard error (SE).

Results

It was calculated that during the entire rearing period, the chickens to which the probiotic preparation was administered at 0.25 g/l received the most *Bacillus subtilis* colonies and the most choline per bird (Table 2). Chickens receiving the same dose of the probiotic preparation (0.25 g/l), but at time T2, received 43% fewer *Bacillus subtilis* colonies and 16% less choline. In the chickens from treatments T1-0.1, T1-0.25 and T2-0.25, the body weight gain was greater than in the G–C group ($P \leq 0{,}027$) (Table 3). In comparison to G–C, the feed conversion rate (FCR) was lower in the chickens from the T1-0.1, T1-0.25 and T2-0.25 treatments ($P \leq 0.035$). Mortality in the experiment was low, at a level of 1–3%. However, it was lower in the experimental groups receiving the probiotic preparation than in group G–C.

Table 3. Body weight gain (BWG), feed conversion ratio (FCR) and mortality rates of chicken receiving the probiotic preparation[1]

Item	Body weight gain (kg)	FCR (kg/kg)	Mortality rate (%)
	1–42 days		
1	2	3	4
Group[2] C	2.608	1.723	3
$T1_{0.05}$	2.665	1.716	2
$T1_{0.1}$	2.672*	1.715*	1
$T1_{0.25}$	2.689*	1.713*	1
$T2_{0.05}$	2.620	1.719	2
$T2_{0.1}$	2.633	1.717	1
$2_{0.25}$	2.650*	1.715*	2
SEM	0.022	0.087	–
Dosage effect (D)			
0.05 (g/L)	2.620	1.718	–
0.1 (g/L)	2.653	1.716	–
0.25 (g/L)	2.669	1.714	–

Table 3 – contd.

1	2	3	4
Time effect (T)			
T1	2.675 a	1.715	–
T2	2.634 b	1.717	–
P–value			
G–C vs. all other	0.027	0.035	–
D effect	0.032	0.052	–
T effect	0.044	0.079	–
D×T interaction	0.332	0.066	–

a, b – means within the same column differ significantly (P≤0.05) according to Newman–Keuls (D×T interaction).

[1]Data represent mean values of 7 replications per treatment. SEM = SD divided by the square root of the replication number, n=7.

[2]Group: G–C = water not supplemented with probiotic: T1-0.05 and T2-0.05 = G–C with probiotic – dose 0.05 g/l; T1-0.1 and T2-0.1 = G–C with probiotic – dose 0.1 g/l; T1-0.25 and T2-0.25 = G–C with probiotic – dose 0.25 g/l; T1-0.05. T1-0.1. T1-0.25 supplemented with probiotic on days 1–42 of life; T2-0.05. T2-0.1. T2-0.25 supplemented with probiotic on days 1–7, 15–21 and 29–35 of life.

*Means within the same column differ significantly from the control (G–C) at P≤0.05 according to Dunnett's mean comparison.

Table 4. Effect of the level and duration of application of the probiotic (*Bacillus subtilis* PB6*)* on redox status in the blood of the chickens[1]

Item		LOOH (µmol/l)	MDA (µmol/l)	SOD (U/ml)	CAT (U/ml)	AST (U/l)	ALT (U/l)
1		2	3	4	5	6	7
Group[2]							
G–C		4.78	0.600	30.06	0.830	219.3	4.95
T1-0.05		3.57*	0.461*	25.82	2.62*	213.9	4.45
T1-0.1		2.55*	0.338*	30.42	2.60*	209.6*	3.82*
T1-0.25		2.014*	0.396*	30.92	2.490*	126.6*	2.58*
T2-0.05		3.95*	0.634*	30.05	0.394*	125.7*	5.69*
T2-0.1		3.87*	0.540*	28.72	0.814	117.3*	4.47
T2-0.25		2.69*	0.530*	28.16	0.773	137.0*	3.93*
SEM		0.114	0.027	0.4583	0.0822	3.814	0.193
Dosage effect (D)	0.05 g/l	63.90 a	0.540 a	27.935	1.507	169.8 a	5.070 a
	0.1 g/l	59.50 ab	0.439 b	29.57	1.707	163.45 a	4.145 b
	0.25 g/l	52.62 b	0.476 b	29.54	1.6315	131.8 b	3.255 b
Time effect (T)	T1	2.71	0.3983	29.053	2.57	183.36	3.616
	T2	3.50	0.5766	28.976	0.6603	126.66	4.696
P-value							
G–C vs. all other		<0.001	0.002	0.257	<0.001	<0.001	0.027
D effect		0.028	0.0428	0.392	0.942	0.0135	0.038

Table 4 – contd.

1	2	3	4	5	6	7
T effect	0.003	0.001	0.456	<0.001	0.003	0.004
D×T interaction	0.053	0.029	0.126	<0.001	0.035	0.453

a, b – means within the same column differ significantly (P≤0.05) according to Newman–Keuls (D×T interaction).

[1]Data represent mean values of 7 replications per treatment. SEM = SD divided by the square root of the replication number, n=7.

[2]Group: G–C = water not supplemented with probiotic: T1-0.05 and T2-0.05 = G–C with probiotic – dose 0.05 g/l; T1-0.1 and T2-0.1 = G–C with probiotic – dose 0.1 g/l; T1-0.25 and T2-0.25 = G–C with probiotic – dose 0.25 g/l; T1-0.05. T1-0.1. T1-0.25 supplemented with probiotic on days 1–42 of life; T2-0.05. T2-0.1. T2-0.25 supplemented with probiotic on days 1–7, 15–21 and 29–35 of life.

*Means within the same column differ significantly from the control (G–C) at P≤0.05 according to Dunnett's mean comparison.

Univariate analysis showed that the level of lipid peroxides in chickens from the T1-0.1, T1-0.25 and T2-0.25 treatments was lower (P<0.001) than in group G–C (Table 4). In all the groups in which the probiotic preparation was used, the MDA level was lower (P≤0.002) than in the control group. Catalase activity was higher in the chickens from the T1-0.05, T1-0.1 and T1-0.25 treatments, but lower in the T2-0.05 group than in the control (P<0.001). AST activity in the plasma of the chickens in all experimental groups was lower (P<0.001) than in the G–C group. In the case of ALT, a similar relationship was observed only for the T1-0.1, T1-0.25 and T2-0.25 treatments. In T2-0.05, ALT activity was higher than in the control (P<0.027). The univariate analysis showed that FRAP was higher (P≤0.035) in the blood of the chickens from all groups receiving the probiotic than in the control group (Table 5). In the chickens from the T1-0.1 and T2-0.25 treatments, the GSH+GSSH level was higher (P≤0.049) than in the G–C group. The plasma level of vitamin C in the chickens from the T1-0.1 and T1-0.25 treatments was higher than in the G–C group (P≤0.023). In contrast, the T2 treatments reduced the content of this vitamin relative to the control (P≤0.023). The level of UA in the blood of chickens from the T1-0.1 and T1-0.25 treatments was lower (P≤0.032) than in the control group, while in the case of T1-0.05 and T2-0.05, it was markedly higher than in the control (P≤0.032). In the blood of the chickens from the T1-0.05 and T10.1 treatments, the BIL level was higher (P≤0.027) than in the control. In the case of CREAT, the value for this parameter was higher (0.042) than in group G–C only in the case of treatment T1-0.25. Univariate analysis showed that the TC level in the chickens from the T2 treatments was higher (P≤0.026) than in G–C (Table 6). In the case of the T1 treatments, there was an increase (P≤0.022) in the proportion of the HDL cholesterol fraction and a decrease (P≤0.045) in that of the LDL fraction relative to the control group. In the blood of chickens from treatment T1-0.05, a decrease (P≤0.013) in TAG was observed as well, and in the case of T1-0.25, an increase (P≤0.006) in NEFA relative to the control. In the blood of chickens from the T1 treatments, there was a decrease (P<0.001) in LDH activity, while in the case of treatments T2-0.05 and T2-0.25, there was an increase compared to the G–C group (Table 6). Analysis of aminotransferase activity revealed that only for the T1-0.05 treatment was there no increase (P<0.001) in ALT

activity relative to the control. GGT activity in the chicken blood from treatments T1-0.05, T1-0.25 and T2-0.25 was lower (P≤0.038) than in the control. Administration of the probiotic preparation resulted in a decrease (P≤0.026) in CK activity in the plasma of chickens from the T1-0.1 and T1-0.25 treatments as compared to G–C. Increased activity of this biocatalyst relative to the control was noted in the case of the T1-0.05 group and all the T2 groups. Compared to the control group, the addition of the probiotic preparation caused an increase (P≤0.033) in HBDH activity in the blood of chickens from treatments T1-0.05, T2-0.05 and T2-0.1, and an increase in AC (P≤0.027) in groups T1-0.05 and T2-0.1.

Dose effect

The use of different doses of the probiotic preparation containing live *Bacillus subtilis* cultures resulted in differences in biochemical parameters in the blood of the chickens.

Table 5. Effect of the level and duration of application of the probiotic (*Bacillus subtilis* PB6) on antioxidant status in the blood of the chickens[1]

Item		FRAP (µmol/l)	GSH+GSSH (µmol/l)	VIT. C (mg/l)	UA (µmol/l)	BIL (µmol/l)	CREAT (µmol/l)
Group[2]							
G–C		88.1	0.057	0.538	154.7	4.88	22.57
T1-0.05		95.2*	0.061	0.556	171.1*	6.76*	21.14
T1-0.1		94.3*	0.068*	0.699*	149.4	6.00*	20.11
T1-0.25		126.8*	0.071*	0.758*	129.6*	4.45	19.8*
T2-0.05		107.8*	0.065	0.158*	190.4*	4.90	22.90
T2-0.1		104.8*	0.066	0.192*	170.0*	4.63	20.31
T2-0.25		98.65*	0.068*	0.246*	156.8	5.17	23.15
SEM		6.85	0.001	0.0155	9.956	0.343	0.785
Dosage effect (D)	0.05 g/l	100.0 b	0.065	0.107	120.5 b	5.83 a	21.67
	0.1 g/l	99.55 b	0.067	0.445	159.7 a	5.31 b	21.63
	0.25 g/l	112.73 a	0.069	0.502	143.2 ab	4.96 b	20.05
Time effect (T)	T1	105.43	0.067	0.671	150.0	5.836	20.52
	T2	103.75	0.066	0.198	172.4	4.90	21.94
P–value							
G–C vs. all other		0.035	0.049	0.023	0.032	0.027	0.042
D effect		0.042	0.062	<0.001	0.041	0.048	0.563
T effect		0.083	0.075	<0.001	0.048	0.035	0.928
D×T interaction		0.072	0.386	0.562	0.325	0.135	0.126

a, b – means within the same column differ significantly (P≤0.05) according to Newman–Keuls (D×T interaction).

[1]Data represent mean values of 7 replications per treatment. SEM = SD divided by the square root of the replication number, n=7.

[2]Group: G–C = water not supplemented with probiotic: T1-0.05 and T2-0.05 = G–C with probiotic – dose 0.05 g/l; T1-0.1 and T2-0.1 = G–C with probiotic – dose 0.1 g/l; T1-0.25 and T2-0.25 = G–C with probiotic – dose 0.25 g/l; T1-0.05. T1-0.1. T1-0.25 supplemented with probiotic on days 1–42 of life; T2-0.05. T2-0.1. T2-0.25 supplemented with probiotic on days 1–7, 15–21 and 29–35 of life.

*Means within the same column differ significantly from the control (G–C) at P≤0.05 according to Dunnett's mean comparison.

Table 6. Effect of the level and duration of application of probiotic (*Bacillus subtilis* PB6) on lipid status in the blood of the chickens[1]

Item		TC (mmol/l)	HDL (mmol/l)	LDL (mmol/l)	TAG (mmol/l)	NEFA (µmol/l)
Group[2]						
G–C		1.85	2.09	1.49	0.46	31.28
T1-0.05		1.57	2.75*	1.04*	0.634*	30.21
T1-0.1		1.62	2.83*	0.82*	0.474	28.25
T1-0.25		1.55	2.74*	0.87*	0.496	25.47*
T2-0.05		4.08*	2.71*	1.18*	0.445	33.69
T2-0.1		3.87*	2.20	1.12*	0.466	31.25
T2-0.25		3.84*	2.32	1.01*	0.445	27.15*
SEM		0.049	0.049	0.055	0.025	24.3
D effect	0.05 g/l	2.82	2.73 a	1.11	0.54 a	63.9 a
	0.1 g/l	2.75	2.51 b	0.97	0.47 b	59.50 ab
	0.25 g/l	2.69	2.53 b	0.94	0.47 b	52.62 b
T effect	T1	1.58	2.41	1.10	0.54	27.98
	T2	3.93	2.77	0.93	0.45	30.70
P-value						
G–C vs. all other		0.026	0.022	0.045	0.013	0.006
D effect		0.082	0.052	0.076	0.042	0.037
T effect		0.035	0.047	0.094	0.052	0.833
D×T interaction		0.324	0.332	0.562	0.484	0.425

a, b – means within the same column differ significantly (P≤0.05) according to Newman–Keuls (D×T interaction).

[1]Data represent mean values of 7 replications per treatment. SEM = SD divided by the square root of the replication number, n=7.

[2]Group: G–C = water not supplemented with probiotic: T1-0.05 and T2-0.05 = G–C with probiotic – dose 0.05 g/l; T1-0.1 and T2-0.1 = G–C with probiotic – dose 0.1 g/l; T1-0.25 and T2-0.25 = G–C with probiotic – dose 0.25 g/l; T1-0.05. T1-0.1. T1-0.25 supplemented with probiotic on days 1–42 of life; T2-0.05. T2-0.1. T2-0.25 supplemented with probiotic on days 1–7, 15–21 and 29–35 of life.

*Means within the same column differ significantly from the control (G–C) at P≤0.05 according to Dunnett's mean comparison.

As the dose of the probiotic increased, the LOOH level in the chicken blood decreased (P≤0.028) (Table 4). In the case of MDA concentration, a dose × time interaction (P≤0.029) was found, which resulted from the fact that the level of malondialdehyde was lower in the chickens receiving the probiotic preparation in the amount of 0.1 or 0.25 g/l, irrespective of the time of administration, which was not noted for the 0.05 g/l dose. A dose × time interaction (P<0.001) was also observed for CAT activity in the blood. At a dose of 0.01 g/l, irrespective of the time of administration, there was an increase in the activity of this enzyme, which was not observed for the other doses (0.05 and 0.25 g/l). For AST activity, there was a dose × time interaction (P≤0.035) resulting from the fact that the effect of the highest dose of the probiotic was manifested as a decrease in AST activity, while no such effect was observed for the remaining doses (0.05 and 0.1 g/l). Higher doses of the probiotic prepara-

tion, i.e. 0.1 and 0.25 g/l, caused a reduction in ALT activity (P≤0.038). The highest dose (0.25 g/l) of the preparation containing live Bacillus subtilis cultures led to the greatest increase in the FRAP index (P≤0.042) and level of vitamin C (P<0.001) (Table 5). In the blood of chickens receiving the probiotic at 0.05 g/l, an increase (P≤0.048) in BIL was observed.

Table 7. Effect of the level and duration of application of the probiotic (*Bacillus subtilis* PB6) on enzymes activity in the blood of the chickens[1]

Item		LDH (U/l)	ALP (U/l)	GGT (U/l)	CK (U/l)	HBDH (U/l)	AC (U/l)
Group[2]							
G–C		497.1	541.2	15.71	411.36	147.36	1.44
T1-0.05		276.6*	558.4	14.22*	479.36*	176.31*	1.69*
T1-0.1		212.5*	724.9*	16.34	369.12*	154.25	1.45
T1-0.25		224.9*	986.0*	13.25*	327.28*	134.15	1.36
T2-0.05		549.6*	1075.2*	14.97	479.36*	169.48*	1.54
T2-0.1		497.0	969.3*	15.69	452.12*	172.31*	1.81*
T2-0.25		593.4*	825.3*	13.77*	461.23*	153.14	1.34
SEM		5.765	40.31	0.29	28.74	9.65	0.124
D effect	0.05 g/l	413.1 a	816.8 b	29.19	958.72 a	345.79 a	3.23 a
	0.1 g/l	354.7 b	847.1 b	32.03	821.24 b	326.56 a	3.26 a
	0.25 g/l	409.15 a	905.6 a	27.02	788.51 c	287.29 b	2.70 b
T effect	T1	238.0	756.4	14.60	391.92	154.90	1.50
	T2	546.6	956.6	14.81	464.24	164.98	1.56
P-value							
G–C vs. all other		<0.001	<0.001	0.038	0.026	0.033	0.027
D effect		0.004	0.006	0.354	<0.001	0.022	0.052
T effect		<0.001	<0.001	0.942	0.003	0.049	0.073
D×T interaction		0.007	0.086	0.775	0.048	0.078	0.092

a, b – means within the same column differ significantly (P≤0.05) according to Newman–Keuls (D×T interaction).

[1]Data represent mean values of 7 replications per treatment. SEM = SD divided by the square root of the replication number, n=7.

[2]Group: G–C = water not supplemented with probiotic: T1-0.05 and T2-0.05 = G–C with probiotic – dose 0.05 g/l; T1-0.1 and T2-0.1 = G–C with probiotic – dose 0.1 g/l; T1-0.25 and T2-0.25 = G–C with probiotic – dose 0.25 g/l; T1-0.05. T1-0.1. T1-0.25 supplemented with probiotic on days 1–42 of life; T2-0.05. T2-0.1. T2-0.25 supplemented with probiotic on days 1–7, 15–21 and 29–35 of life.

*Means within the same column differ significantly from the control (G–C) at P≤0.05 according to Dunnett's mean comparison.

The use of higher doses of the probiotic, i.e. 0.1 g/l and 0.25 g/l, resulted in a decrease in the plasma concentrations of NEFA (P≤0.037) and TAG (P≤0.042) in the chickens (Table 6). In the case of LDH activity, a dose ´ time interaction (P≤0.007) was found; a strong effect of the middle dose (0.1 g/l) of the probiotic was manifested as a decrease in the activity of this enzyme, which was not observed with the other two doses (0.05 and 0.25 g/l) (Table 7). As the amount of the probiotic prep-

aration used was increased, ALP activity increased in the chicken serum ($P \leq 0.006$). In the case of CK activity, a dose × time interaction ($P < 0.001$) resulted from the fact that the activity of this enzyme was much lower in the blood of chickens receiving the highest probiotic dose (0.25 g/l), irrespective of the time of administration, than in those receiving other doses. In the blood of chickens receiving the highest dose of the preparation containing live *Bacillus subtilis* cultures (0.25 g/l), a reduction in HBDH activity ($P \leq 0.022$) was observed relative to the groups receiving lower doses of the probiotic (0.05 and 0.1 g/l).

Time effect

The use of two different modes of administration of a probiotic preparation containing live *Bacillus subtilis* cultures (continuous and periodic) led to differences in indicators of antioxidant and redox status, the lipid profile, and the activity of selected enzymes in the blood of broiler chickens.

Irrespective of the dose of probiotic used, the longer application time (T1) resulted in a decrease ($P \leq 0.003$) in the plasma concentration of LOOH and MDA in the chickens (Table 4). Administration of the probiotic at time T1 caused a greater reduction in ALT activity ($P \leq 0.004$) than the use of the preparation at time T2. Analysis of the level of indices of antioxidant status revealed that the level of vitamin C in the blood of chickens receiving the probiotic during time T1 was higher ($P < 0.001$) than in birds treated with the probiotic during time T2 (Table 5). The longer duration of administration (T1) also caused a reduction in UA ($P \leq 0.048$) and an increase in BIL ($P \leq 0.035$) relative to time T2. The use of the probiotic preparation at time T1 reduced the level of TC ($P \leq 0.035$) more than administration at time T2 (Table 6). The use of the probiotic at time T2 significantly increased activity of ALP ($P < 0.001$) and HBDH to a much greater extent than administration at time T1 ($P \leq 0.049$) (Table 7).

Discussion

The use of a probiotic preparation containing live cultures of *Bacillus subtilis* enriched with choline at the highest dose (0.25 g/l), continuously for 42 days, resulted in the highest final body weight and the lowest FCR. Most likely this probiotic regimen allowed for the most successful colonization by the probiotic strain, which contributed to the most satisfactory growth performance. Increased weight gain, lower FCR, and reduced mortality following the use of *Bacillus subtilis* in chicken diets have also been reported by Haque et al. (2017), Tang et al. (2017) and Zhang and Kim (2014).

It is worth noting that the group with the highest final body weight was the one receiving the largest addition of choline from the probiotic preparation. According to Waldroup et al. (2006), better growth performance following the use of choline is due to its stimulating effect on absorption of essential amino acids in the intestines (Waldroup et al., 2006). According to Zeisel and da Costa (2009) choline from the feed ration is used to a great extent for the production of methionine, an essential

amino acid that determines more efficient utilization of other dietary components. It is worth noting that the use of similar doses of *Bacillus subtilis* colonies and choline during the rearing period, but at different times, resulted in similar growth performance (Waldroup et al., 2006).

Our results indicate that the highest dose of the probiotic preparation, especially when administered continuously, had the most beneficial effect, which was manifested as a reduced concentration of compounds characterizing unfavourable oxidation processes, i.e. LOOH and MDA, as well as an increased level of substances indicative of antioxidant capacity: FRAP, GSH+GSSH and vitamin C. According to Capcarova et al. (2010), the use of a probiotic preparation containing live *Bacillus subtilis* cultures and choline may favourably increase antioxidant capacity by improving the resistance of biological macromolecules to oxidation and neutralization of hydroxyl radicals. Our results are in agreement with reports by Ognik et al. (2017), who found a decrease in the level of LOOH and MDA in the blood of chickens receiving a probiotic containing live *Enterococcus faecium* cultures. MDA and LOOH are biological markers of oxidative stress, and as the main products of peroxidation of polyunsaturated fatty acids they provide information regarding the degree of damage to cell membranes (Naaz et al., 2014). A reduction in MDA and LOOH levels is indicative of high efficiency of the enzyme antioxidant defence system (Ognik et al., 2016) and the resistance of important biological molecules to oxidation (Zheng et al., 2016). According to Wang et al. (2017), probiotic microbes have their own antioxidant systems and can stimulate the functionality of antioxidants in the host. Bacterial strains with such properties include *Lactobacillus fermentum*, *Lactobacillus acidophilus*, *Bifidobacterium lactis* and *Bacillus amyloliquefaciens* (Wang et al., 2017). According to Pajare et al. (2018), LOOH can also increase lipid peroxidation, not only directly by supplying lipid radicals, but also indirectly by accelerating oxidation of Fe^{+2} to Fe^{+3}. According to the author, LOOH generated during peroxidation are able to oxidize Fe^{+2}, thus unfavourably altering the Fe^{+2}/Fe^{+3} ratio. When the concentration of Fe^{+2} reaches a sufficiently low level, lipid peroxidation begins in the liposomes, and the increased level of Fe^{+3} is conducive to very rapid initiation of lipid peroxidation expressed as synthesis of LOOH. In addition, autoxidation of Fe^{+2} is associated with the production of superoxide anion radical and hydrogen peroxide (Pajare et al., 2018).

In our study, continuous administration of the probiotic preparation increased CAT activity without affecting SOD activity in the plasma, which was not observed in the case of periodic administration of the same doses. The increase in CAT activity should be considered a beneficial phenomenon, because it may suggest a lower intensity of stress reactions in cells (Ognik and Krauze, 2016). According to Rajput et al. (2013), probiotics can stimulate the endogenous antioxidant defence mechanism, thereby preventing the effects of oxidative stress. In a study using *Bacillus subtilis* in chicken rearing, Rajput et al. (2013) found that the bacteria can protect molecules against oxidation, help to remove hydroxyl radicals, and improve antioxidant status. According to Ognik and Krauze (2016), this outcome indicates strong mobilization of the body's defence mechanisms. Oxidative stress and increased production of reactive oxygen species in the mitochondria lead to activation of cellular antioxidant

defence, resulting in reduced CAT and SOD activity and lower GSH levels (Ognik and Krauze, 2016; Ognik et al., 2016). Rajput et al. (2013) and Yener et al. (2009), following administration of probiotics containing *Saccharomyces boulardii* or *Bacillus subtilis*, observed a simultaneous increase in CAT and SOD activity, which according to Ognik and Krauze (2016) and Ognik et al. (2016) indicates a strong mobilization of the body's defence mechanisms under stress conditions. In our research, only periodic application of the probiotic preparation containing live *Bacillus subtilis* cultures and choline resulted in a decrease in CAT activity in the blood.

According to Farina et al. (2017), the increased level of GSH+GSSH observed in our study can be explained by the beneficial effect of choline on glutathione synthesis. According to Zeisel and da Costa (2009), choline deficiency increases lipid peroxidation in the liver, and the lipid peroxides generated in this process, as a potential source of free radicals, cause changes in DNA, activating the carcinogenesis process. Insufficient intake of choline leads to destruction of mitochondrial membranes and slows down β–oxidation of fatty acids, and by reducing respiratory chain activity it disturbs energy production in the mitochondrion (Serviddio et al., 2011). According to Metha et al. (2009), choline supplementation supports phosphatidylcholine biosynthesis, reduces oxidative stress by inhibiting lipid peroxidation and increasing total antioxidant capacity, and indirectly increases glutathione levels.

The reduction in aminotransferase activity observed in the chicken blood supports the results of research by Abudabos et al. (2017), who also used *Bacillus subtilis* in chicken diets. AST is common in the skeletal muscles, heart, and liver, whereas the original source of ALT is primarily the liver. According to Haque et al. (2017), significantly increased aminotransferase activity could suggest cell toxicity caused by lipid peroxidation of cell membranes. In addition, according to Farina et al. (2017), choline activates enzymatic detoxification reactions, thereby supporting liver function, and is effective in treating various liver diseases. As a component of phosphatidylcholine, it has a lipotropic effect, preventing the deposition of fat and cholesterol in this organ (Farine et al., 2017).

The analysis of lipid status indicators enabled comparison of the results with those reported by other researchers who have also assessed the effect of probiotics on lipid metabolism (Sobczak and Kozłowski, 2015; Ognik and Krauze, 2016; Tang et al., 2017). Tang et al. (2017) noted an increase in the HDL fraction and a reduction in LDL in the total cholesterol pool after administering a probiotic containing strains of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, *Streptococcus faecium* and *Aspergillus oryzae* to chickens. According to Hooper et al. (2001), when intestinal bacteria synthesize hydrolases they stimulate fat metabolism in the liver, thereby indirectly affecting the metabolism of cholesterol and fatty acids. In our study, the use of a probiotic preparation containing *Bacillus subtilis* throughout the rearing period resulted in a beneficial reduction in TC in the blood. This effect may also have been influenced by the addition of choline to the probiotic preparation. This compound increases synthesis of L–carnitine, which is necessary for the transport of fatty acids oxidized during β–oxidation (Farine et al., 2017). On the other hand, periodic administration of all doses caused an unfavourable increase in the content of this lipid in the blood of the chickens. A decrease in

the level of TC in the blood of chickens following the use of *Bacillus subtilis* has also been reported by Sobczak and Kozłowski (2015), Haque et al. (2017) and Fathi et al. (2018). A reduction in TC has also been observed by Sohail et al. (2010), who used an entire group of strains of probiotic bacteria: *Lactobacillus plantarum*, *Lactobacillus delbrueckii*, *Bulgaricus*, *Lactobacillus acidophilus, Lactobacillus rhamnosus*, *Bifidobacterium bifidum* and *Streptococcus salivarius*.

In our study, the decrease in the level of TAG following administration of *Bacillus subtilis* is consistent with results obtained by Rajput et al. (2013), who used various *Bacillus* strains in chicken diets. Zhang et al. (2014) achieved a similar effect after giving chickens *Lactobacillus* strains. According to Ognik et al. (2017), the reduction in TC and TAG concentrations and the share of LDL cholesterol following administration of probiotics is due to a reduction in oxidative processes in the cell, which is accompanied by an increase in these parameters (Ognik and Krauze, 2012). The content of cholesterol and phospholipids and the degree of fatty acid saturation is a crucial factor for the fluidity of the cell membrane, and according to Fki et al. (2007), observation of the level of TAG, TC and its fractions can be successfully used to assess the intensity of lipid oxidation, which is accompanied by hypercholesterolaemia (Fki et al., 2007). The reduction in TC levels can be explained by the ability of some bacterial strains to incorporate cholesterol into their cells and to inhibit the activity of hydroxymethylglutaryl–CoA reductase, which is necessary in the cholesterol synthesis pathway, thus slowing down synthesis of this compound. In addition, according to Tang et al. (2017), the mechanism by which probiotics reduce TC and TAG levels may result from their properties increasing enzymatic destruction of cholesterol molecules (deconjugation), which is catalysed by bile salt hydrolase. In contrast, Mohebbifar et al. (2013) observed no significant impact of a probiotic supplement on the level of TC and TAG in chicken blood. According to Pourakbari et al. (2016), the use of probiotics in chickens increases levels of TC and HDL and reduces LDL, which according to these authors improves the lipid status of the blood. Despite discrepancies in the interpretation of results, most authors indicate a beneficial effect of probiotic strains on the level of lipid indices. In addition, according to Schenkel et al. (2015), choline may reduce TC and TAG levels and significantly increase the share of HDL. By facilitating the transport of TC to the cells, choline reduces its level in the blood, which helps to maintain the proper structure and function of biological membranes (Schenkel et al., 2015).

Under stress conditions, metabolic changes and stimulation of lipolysis may occur, accompanied by an increase in NEFA. The reduction in NEFA obtained in our research can be considered a beneficial effect, which according to Verago et al. (2001) may be due to the body's better adaptation to the prevailing conditions or to a decrease in the stress response following administration of *Bacillus subtilis*. An increase in NEFA is a consequence of inhibition of triacylglycerol synthesis due to the utilization of glycerol to synthesize glucose in the gluconeogenesis process (Verago et al., 2001). As in our research, a reduction in UA and CREAT levels has been observed by Siadati et al. (2017), who used probiotics containing *Lactobacillus* strains in the diet of Japanese quail, and by El-Faham et al. (2014), who used this genus in broilers. Probiotics most likely improve protein metabolism and kidney function,

which enables better utilization of nitrogen, and according to Salim et al. (2011), UA and CREAT, as well as other toxins, can be used as nutrients for the growth of probiotic bacteria. According to Fathi et al. (2013), low CREAT levels may indicate an improvement in protein metabolism and a protective effect of probiotics on kidney function.

Conclusions

The study demonstrated that the dose and time of administration of a probiotic containing live *Bacillus subtilis* PB6 cultures enriched with choline are associated with improvement in the body's antioxidant defences. These beneficial changes were most evident following the dose of 0.25 g/l administered to chickens during the entire 42–day rearing period (T1). At that time, each bird received 33.3 CFUx10^{11} *Bacillus subtilis* from the probiotic preparation. In this case, there was a beneficial increase in total antioxidant potential and a reduction in the level of stress indicators, while metabolic processes remained undisturbed. The dose of 0.25 g/l applied at time T1 had the most beneficial effect on the growth performance of the chickens.

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