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To Whom It May Concern:

This is to confirm that the manuscript PAAP-D-17-00276 entitled "Bacilli probiotics supplementations improve laying performance, eggs quality, hatching of laying 2 hens and sperm quality of roosters" by Maria S. Mazanko, Ivan F. Gorlov, Evgeniya V. Prazdnova, Maxim S. Makarenko, Alexander V. Usatov, Anzhelika B. Bren, Vladimir A. Chistyakov, Alexey V. Tutelyan , Zoya B. Komarova, Natalia I. Mosolova, Denis N. Pilipenko, Olga E. Krotova, Aleksandr N. Struk, Angela Lin, and Michael L. Chikindas was submitted to Probiotics and Antimicrobial Proteins on November 19, 2017. The manuscript was reviewed by two specialists in the field of the study and was suggested for publication after minor revision (November 28, 2017).

Michael Leonidas Chikindas, Ph.D.

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Bacilli probiotics supplementations improve laying performance, eggs quality, hatching of laying hens and sperm quality of roosters

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Bacilli probiotics supplementations improve laying performance, eggs quality, hatching of laying hens and sperm quality of roosters

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Running head title: Bacilli probiotics benefit poultry

Abstract

The study aims at elucidating the effect of bacilli probiotic preparations on the physiology of laying hens and roosters. Probiotic formulations were prepared as soybean products fermented by *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquefaciens* B-1895. In this study, groups of male and female chickens were used. These groups received a probiotic preparation based on either *B. subtilis* KATMIRA1933 or *B. amyloliquefaciens* B-1895, or of a mixture of strains, from the first day to the age of 39 weeks. These preparations positively affected egg production, quality of sperm production, and quality and hatchery of eggs. Considering the simplicity and cost-effectiveness of the soy-based probiotic preparation, these formulations should be considered as advantageous in modern livestock production.

Key world: probiotic; Bacillus; poultry; egg production; sperm quality

Introduction

Poultry is one of the most important sources of protein (meat and eggs) for humans. Due to the growing demand for food products over the past few years, poultry production has increased significantly [1].

Internationally, antibiotics such as tetracycline, amoxicillin, penicillin, bacitracin, and more are used routinely as a chicken growth promoter and as a preventive antimicrobial measure [2]. However, the use of antibiotics in poultry farming leads to the spread of antibiotic resistance and the development of microbiota disturbances in birds [2, 3]. For these purposes, probiotics should be considered as an alternative to antibiotics [4]. The World Health Organization defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [5]. Similar to antibiotics, some probiotics inhibit the growth of microbial pathogens in the intestines of birds, thus reducing morbidity. Moreover, probiotics do not trigger antibiotic resistance in the gut bacteria and their use do not lead to the accumulation of toxic antibiotics in bird tissues [6, 7].

Most of the probiotic microorganisms used in poultry farming belong to *Lactobacillus spp.*, *Bifidobacterium spp.*, and *Enterococcus spp*. They are utilized either as monocultures or in multi-species formulations. Additionally, there is a noticeable increase in the use of bacilli based probiotic formulations in poultry farming. *Bacillus* species are suitable feed additives because of their spores' stability and ability to produce a variety of enzymes such as protease, amylase, and lipase [8].

Materials and methods

The research was carried out according to the approved conditions at JV "Svetly", which is a structural unit of CJSC "Agrofirma" Vostok "(Volgograd region, Russia), the sow farm of the second order for poultry breeding "Highsex brown".

Probiotics

Two strains of probiotic bacteria were used: *B. subtilis* KATMIRA1933, the fermented milk product isolate [9] and *B. amyloliquefaciens* B-1895, the soil-derived microorganism.

The protocol for solid-phase fermentation of probiotic bacilli was described in detail in our study [10]. Briefly, bacterial strains were inoculated on plates with solid LB medium (Difco, MI) and incubated for 1 day at 37°C. Soy beans (1 kg) were washed with running water, soaked for 12 h at room temperature, sterilized at 115°C for 40 min, placed in an incubator and cooled to 60°C. The soy bean preparation was inoculated with the biomass of bacteria from one plate, mixed thoroughly and incubated for 24 h at 42°C aerobically. The fermented substrate was milled with a meat grinder, distributed in a thin layer on metal trays, and dried at 50°C to a humidity of 8-10%. Viable cells were enumerated at each step of the process by seeding on the appropriate solid medium.

***In vivo* experimental procedures**

Parent herd of the "High-sex brown" cross (hatched on August 25, 2016) was obtained from the Sverdlovsk PPR Ltd. (Sverdlovsk Region). Eight groups of one day-old chicks were formed: 4 groups of female chickens with 70 animals per group and 4 groups of male chickens with 7 animals each. These groups consisted of a control and experimental (I, II, and III) sub-groups. The control group received a standard diet, while experimental animals received the diet with probiotic strains (group I received a probiotic preparation based on the *B. subtilis* strain KATMIRA1933, group II received a probiotic preparation based on the strain *B. amyloliquefaciens* B-1895 and group III received a probiotic preparation based on the mixture of the two bacilli strains).

These preparations were introduced into the diet as additives. Additive №1 included a probiotic preparation based on the *B. subtilis* strain KATMIRA1933 (10^7 – 10^9 CFU/g viable spores) and extruded pumpkin press cake (included in the main diet) as a filler. Additive №2 included a probiotic preparation based on the strain *B. amyloliquefaciens* B-1895 (10^7 – 10^9 CFU/g viable spores) and extruded pumpkin press cake as a filler. Additive №3 included probiotic preparation based on *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 (equal amounts, 10^7 – 10^9 CFU/g viable spores) and extruded pumpkin press cake as filler.

Doses of the preparations' administration were 1% in the overall structure of the poultry diet, and the dose of probiotic supplements was 0.1%.

Each experimental bird was contained in the cell battery Big Dutchman (Germany). The microclimate parameters were set according to the recommendations of the manufacturer of cross-country "High-sex brown" company "ISA Hendrix Genetics" (Holland).

The birds were fed with the standard mixed fodder manufactured at the feed mill of the company. Feeding of the experimental birds was carried out according to NRC [11]. Weighing of the experimental young animals was carried out on the weekly basis. The conversion of the feed was calculated as the ratio of the weight of the expended feed to the weight gain of the bird.

Quality of sperm

Semen from the birds was collected by abdominal massage [12] and evaluated for the selected gross semen variables such as semen volume, sperm concentration, and live and abnormal sperm.

Sperm viability and abnormality were evaluated using a portion of ejaculate stained with an eosin-nigrosin solution. The stained seminal smears were prepared in duplicates and 200 sperm per slide were evaluated for viability, where unstained spermatozoa were considered as live. Spermatozoa with detached heads, abaxial heads, malformed heads, bent tails, coiled tails, double tails, and protoplasmic droplets were considered as abnormal, as described [13, 14].

Sperm concentration was determined in duplicate, using a Neubauer hemocytometer [14].

Egg production and quality of eggs

Egg production was calculated using the following formula:

$$\text{Hen} - \text{Day Egg Production (HDEP)} = \frac{\text{Total number of eggs produced during the period}}{\text{Total number of hen} - \text{days in the same period}} * 100\%$$

Haugh unit (H.U.) was calculated using the formula:

$$H.U. = 100 * \log(h - 1.7w^{0.37} + 7.6)$$

where *h* is albumen height in millimeters, measured by spherometer and *w* is the observed weight of the egg in gram [15].

The eggs' length and breadth were measured with digital caliper and the shape index was calculated as the ratio of breadth to length x 100.

Albumen weight was calculated as egg weight - (yolk weight + shell weight). Albumen and yolk ratios were calculated taking their individual weights as the percentage of the total egg weight. Albumen and yolk indices were estimated as a percentage, taking the ratio of their respective heights to the average of breadth and length as suggested in previously published reports. Yolk albumen ratio was calculated as the weight of yolk/weight of albumen [16, 17].

Hatchability was calculated as the percentages of all the eggs set that hatched.

Statistical processing of experimental data

The statistical significance of the differences was determined by the Student's *t*-test for independent samples at $p < 0.05$.

Ethics of biological experiments

Experiments on animals were conducted in accordance with the principles of the European Convention for the Protection of Vertebrate Animals, used for experiments or for other scientific purposes.

Results

Quality of rooster sperm production

In pedigree roosters the males of the experimental groups exceeded the control volume of the ejaculate, the spermatozoa concentration, and the total number of spermatozoa in the ejaculate. The number of morphologically abnormal cells in the ejaculate of the roosters of the experimental groups decreased (Table 1).

Egg production

The age of the first egg-laying was found to be dependent on the reproductive organ development which was followed during the pullet production. In the second and third experimental groups, the first egg was laid at the age of 126 days, in the control group at 127 days, and in the first test group at 128 days. The poultry productivity in all experimental groups during the first five months of oviposition (39 weeks) was higher than in the control group (Table 2, Figure 1).

At the age of 39 weeks, the birds of all the groups reached the peak of productivity. However, during the entire period of observations, the number of laid eggs in the first experimental group was higher than in the test groups II and III by 69 and 56 more eggs respectively, and it measured 119 eggs more than the control group.

Hatching egg quality

For the study's purposes, the eggs were incubated from the 28 weeks old birds. Prior to the incubation, morphological and chemical analyses of the eggs were conducted (Table 3).

Morphological analysis of incubation eggs showed that the weight of eggs in all experimental groups exceeded the control. The increase of the eggs mass was due to the mass of the yolk.

The protein index and the number of Haugh unit in the experimental groups were significantly higher than those of the control. The thickness of the eggshell in experimental groups exceeded the control, too. The chemical composition of the experimental laying hens' eggs was within the physiological norm and did not differ significantly from the eggs in the control group.

Egg hatchability

Poultry is characterized by high reproductive qualities, which are determined by a number of factors such as the intensity of laying, high fertilization, and hatchability of eggs. Egg hatchability characterizes the biological fullness of fertilized eggs and the viability of embryos and hatched young animals. Our results indicate that in all experimental groups the output of the chickens was high and corresponded to the standard characteristic to the cross (Table 4).

However, in experimental group I, the hatching rate exceeded the control by 2.14%, with 84.64 against 82.50 in the control. In the group II, the observed excess in hatching was 1.43%, and it reached just 0.71% in the experimental group III (almost equivalent to control). The higher yield of chicks in the experimental groups was obtained by increasing the eggs fertilization and reducing the number of embryo deaths during the first 7 days of incubation. This indicates a biological incorporation of the bacilli from the feed that stems from the hen to their young.

Discussion

According to the literature, probiotics affect numerous parameters in hens and eggs. These include biochemical blood indices showing the intensity of carbohydrate and protein metabolism (protein, glucose, urea content); hematological composition of blood (number of blood corpuscles); dynamics of live weight (weight gain); conversion rate of feed (apparently, it is increased by improving digestion and absorption of nutrients, leading to better performance); quantitative and qualitative composition of the microbiota; the level of oxidative stress (mRNA expression of antioxidant genes, oxidative damage index, etc.); meat quality (pH, drip loss, cooking loss, shear force, color); laying performance; egg quality (yolk cholesterol level, improved shell thickness, egg weight); intestinal barrier function of laying hens [8, 18, 19, 20].

In our study, the introduction of probiotic bacteria into the diet of birds led to the increase in sperm production, egg production, egg quality and hatchability. We speculate that these qualities resulted from the production of a large number of lytic enzymes and metabolites exhibiting antioxidant and DNA-protective properties by the studied strains [21]. The observed effects can also be due to the bacilli-produced proteases, amylases and cellulases which contribute to the better digestion of the feed.

Probiotics strains of *Lactobacillus, Streptococcus, Bacillus, Bifidobacterium, Enterococcus, Aspergillus, Candida* and *Saccharomyces* species have been shown to increase resistance of chickens to *Salmonella*, *E. coli* and *Clostridium perfringens* infections. In addition, oral inoculation of *Bacillus subtilis* spores reduced intestinal colonization of pathogenic *E. coli* in chickens [18, 22].

The use of bacilli-based probiotic formulations also seems to be a promising health-promoting approach. *Bacillus* spp. are widely used in the poultry industry [23, 24, 35]. They demonstrate adaptability to diverse conditions and long shelf life. *Bacillus* spp., including *B. amyloliquefaciens* can be found in the normal intestinal microbiota and are capable of germinating and re-sporulating in the gastrointestinal tract [24, 26, 27, 28, 29]. Moreover, their ability to form biofilms is important for functionality as a medical and veterinary probiotic [30].

Noticeably, probiotics affect the characteristics of the laid eggs. *Enterococcus faecium* supplementation was shown to result in a significant increase in egg production, eggshell thickness, and nutrient digestibility in laying hens, and a decrease in fecal coliform counts [31].

Data on the impact of probiotic on the egg production are somewhat contradictory. For instance, hens fed with 0.01% and 0.06% of *B. licheniformis* had improved egg production over control group (98.4% and 94.0%, respectively) [8]. Kurtoglu *et al*. [32] showed that the hens fed with up to 750 mg of probiotic (3.2×10^9 cfu/g)/kg of diet had improved egg production, whereas Li *et al*. [33] and Yalcin *et al*. [34] demonstrated no statistically significant effect of probiotics on hen egg production. These effects seem to be strain-specific.

In the present study, we observed a similar situation: the number of laid eggs significantly increased, as well as their quality. In addition, the quality of the sperm of roosters improved.

Probiotic supplementation may be even more effective in stress conditions than in normal. Thus, Jia *et al*. showed that *B. subtilis* reduced the adverse effects of mycotoxins on laying performance, effectively improving egg quality and reducing the accumulation of aflatoxins residues in the egg [35].

Based on the data presented here, it can be concluded that the use of probiotic preparations based on the *Bacillus subtilis* KATMIRA1933 and Bacillus *amyloliquefaciens* B-1895 positively affect the rate of growth and condition of the birds, both the rearing flocks and the laying hens. The weight, egg production, egg quality and hatchery increase. Considering the simplicity and economical effectiveness of the studied fermented soybeans-based probiotic preparations, the use of these formulations can present some benefits for the modern livestock production.

The ongoing investigation is dedicated to the observation of the birds' conditions, productivity and incubatory qualities of eggs with the duration of the study extended up to 45-50 weeks.

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Conflict of interests

The authors declare no conflict of interests.

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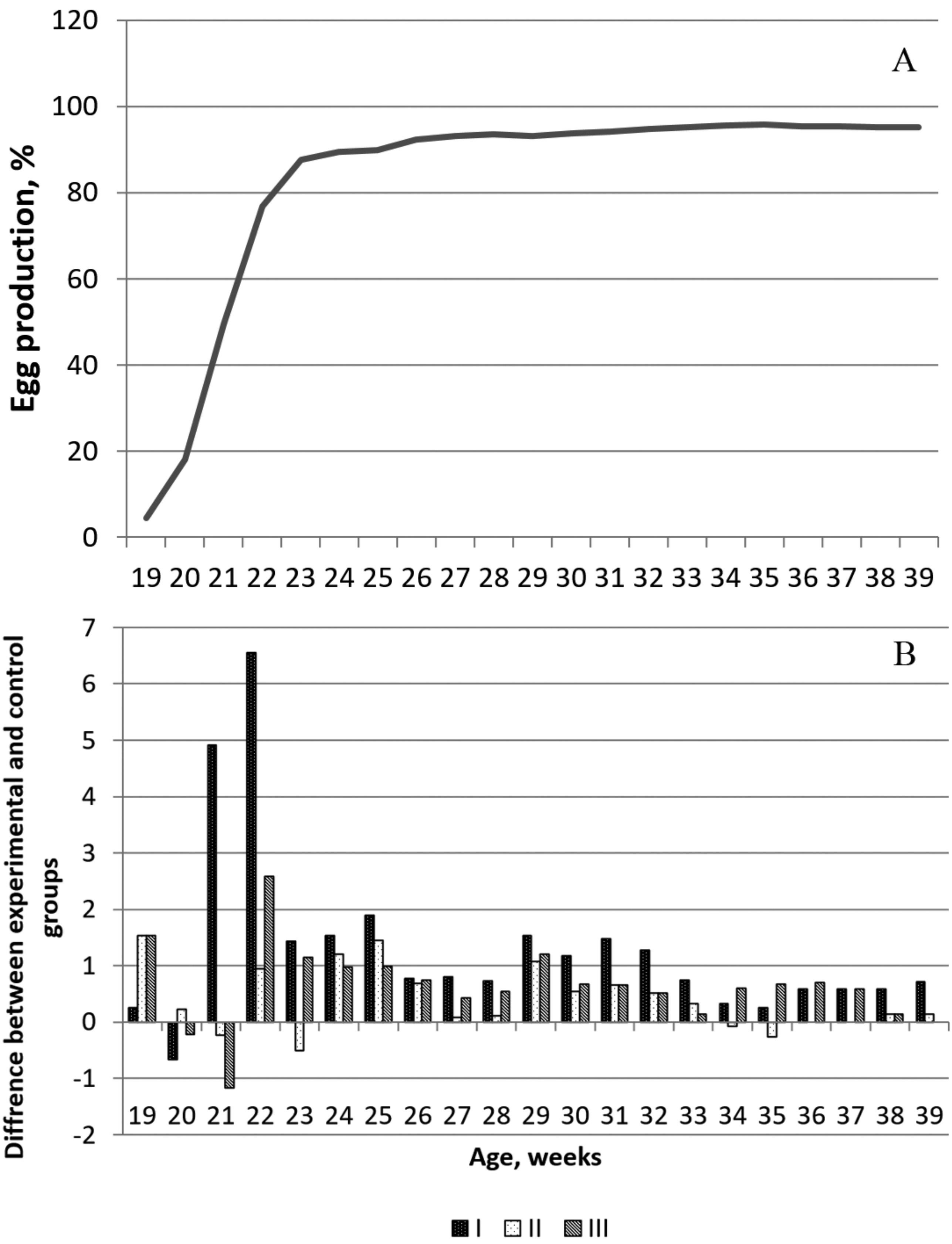
Figure legends

Fig. 1. Egg production of control group birds (A) and the difference in egg production of the experimental groups from the control group (B), %.

Fig. 1.



A
Egg production, %
120
100
80
60
40
20
0
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
B
Diffrence between experimental and control groups
7
6
5
4
3
2
1
0
-1
-2
Age, weeks
I
II
III

Table 1. Quality of the rooster sperm production (n = 5).

Index	Group			
	control	experimental I	experimental II	experimental III
Color	white	white	white	white
Volume of ejaculate, ml	0.50 ± 0.04	0.56 ± 0.03	0. 53 ± 0.04	0.54 ± 0.05
Total number of spermatozoa in the ejaculate, 10^9	1.49 ± 0.05	1.75 ± 0.06*	1.61 ± 0.04	1.69 ± 0.06
Concentration of spermatozoa, 10^9/ml	2.56 ± 0.08	3.29 ± 0.07**	3.01 ± 0.09*	3.17 ± 0.09**
The number of morphologically abnormal germ cells in the ejaculate, %	14.7 ± 0.40	10.4 ± 0.51**	11.7 ± 0.43**	10.1 ± 0.62**

Table 2. The number of eggs laid by the control and test groups up to the age of 39 weeks.

	control	experimental I	experimental II	experimental III
Number of chickens from 19 to 21 weeks	64	64	64	64
Number of chickens from 22 to 39 weeks	61	61	61	61
Number of eggs, pcs.	7,419	7,538**	7,469**	7,482**
Difference with the control, pcs.	-	119**	50**	63**
% of control	-	101.6**	100.7**	100.8**

* Beginning of egg-laying - 19 week

**Differences are statistically significant, paired *t*-test, $p < 0.01$

Table 3. Morphological indices of the hatched eggs (n = 10).

Index	Groups			
	control	experimental I	experimental II	experimental III
Egg weight, g	61.64 ± 0.42	63.49 ± 0.67*	62.87 ± 0.49	63.11 ± 0.37*
Weight of egg parts, g: albumen	36.48 ± 0.29	37.15 ± 0.31	37.00 ± 0.27	37.06 ± 0.40
yolk	18.89 ± 0.17	19.55 ± 0.19*	19.26 ± 0.15	19.32 ± 0.13
shell	6.2 7± 0.09	6.79 ± 0.08**	6.61 ± 0.07*	6.73 ± 0.08**
Shape index, %	75.93 ± 0.51	75.04 ± 0.43	75.92 ± 0.32	75.18 ± 0.64
Albumen index, %	9.12 ± 0.14	9.92 ± 0.16**	9.68 ± 0.11*	9.84 ± 0.15**
Yolk index , %	44.85 ± 0.69	48.83 ± 0.54**	48.18 ± 0.61**	48.51 ± 0.47**
Haugh unit	81.47 ± 0.27	82.92 ± 0.33**	82.67 ± 0.28*	82.81 ± 0.36*
Shell thickness, μm	358.00 ± 2.14	370.00 ± 2.28**	365.00 ± 2.11*	368.00 ± 1.99*
Ratio of egg parts, %: albumen	59.18 ± 0.27	58.51 ± 0.14	58.85 ± 0.13	58.72 ± 0.17
yolk	30.65 ± 0.18	30.79 ± 0.15	30.63 ± 0.17	30.61 ± 0.21
shell	10.17 ± 0.04	10.69 ± 0.06	10.51 ± 0.05	10.66 ± 0.06
Ratio of albumen/yolk	1.93 ± 0.015	1.90 ± 0.018*	1.92 ± 0.014	1.92 ± 0.013

Table 4. Results of the egg incubation.

Index	Groups							
	control		experimental I		experimental II		experimental III	
	number	%	number	%	number	%	number	%
Eggs laid in the incubator	280	100	280	100	280	100	280	100
Fertility of eggs	260	92.86	264	94.29	262	93.57	263	93.93
Incubation waste, incl.:								
unfertilized eggs	20	7.14	16	5.71	18	6.42	17	6.07
“blood ring”	12	4.29	10	3.57	9	3.21	10	3.57
dead-in-shell	9	3.21	10	3.57	11	3.93	13	4.64
late dead	8	2.86	7	2.51	7	2.51	7	2.51
Hatching rate, heads	231	-	237	-	235	-	233	-
Healthy hatched chicks, %	-	82.50	-	84.64	-	83.93	-	83.21
Eggs hatchability, %	-	88.85	-	89.77	-	89.69	-	88.59