Kocatepe Veterinary Journal

Kocatepe Vet J (2020) 13(2):145-151

DOI: 10.30607/kvj.695761

RESEARCH ARTICLE

The Effect of Dietary Probiotic Supplementation on Egg Weight in Laying Hens: A Meta–Analysis Study

İbrahim KILIÇ[1]*, İhsan BERK[2], Zehra BOZKURT[3], Yağmur Nil DOĞAN[4]

[1]Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Biostatistics, 03200, Afyonkarahisar, Turkey
[2]Afyon Kocatepe University, Faculty of Sciences, Department of Statistics, 03200, Afyonkarahisar, Turkey
[3]Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Animal Husbandry, 03200, Afyonkarahisar, Turkey
[4]Gaziantep University, İslahiye Vocational School, Department of Veterinary, 27000, Gaziantep, Turkey

ABSTRACT

The aim of this study was to determine the effect of probiotic supplementation on egg weight in laying hens by using meta–analysis. The limitations of the eight studies –indexed in SCI– taken into the meta–analysis were 18 – 42 week old brown and white laying hens; 10^7 – 10^{10} CFU/g *Bacillus subtilis* was used as a probiotic and the dose ranged between 400 – 1000 g/ton. The meta–analysis was carried out using (experimental–control) means for continuous data. As a result of the research, it was determined that all studies did not share a single common effect (heterogeneous). In addition, the overall effect size using the random effect model was calculated as 0.223. Based on z and p values, the hypothesis of the study was accepted ($z = 2.90$; $p < 0.05$). In other words, "the probiotic has a significant effect on egg weight in laying hens".

Keywords: Probiotic, *Bacillus subtilis*, egg weight, laying hens, meta-analysis

***

Yumurtacı Tavuklarda Diyet Probiyotik Takviyesinin Yumurta Ağırlığına Etkisi: Bir Meta Analizi Çalışması

ÖZ

Bu çalışmanın amacı, yumurtacı tavuklarda diyet probiyotik takviyesinin yumurta ağırlığı üzerindeki etkisini meta-analiz kullanarak belirlemektir. Meta analize alınan sekiz çalışmanın sınırlılıkları; SCI-exp endeksinde olması, 18-42 haftalık kahverengi ve beyaz yumurtlayan tavukların kullanılması; probiyotik olarak 10^7-10^{10} CFU / g *Bacillus subtilis* kullanılması ve doz olarak 400-1000 g / ton arasında değişmesiydi. Meta-analizinde, sürekli veriler için (deneysel-kontrol) ortalamalar kullanılarak etki büyüklüğü hesaplanmıştır. Araştırma sonucunda, tüm çalışmaların tek bir ortak etkiyi paylaşmadığı belirlendi. Ayrıca, rastgele etki modeli kullanılarak toplam etki büyüklüğü 0.223 olarak hesaplandı ve z ve p değerlerine göre çalışmanın hipotezi kabul edildi ($z = 2.90$; $p <0.05$). Başka bir ifadeyle; "yumurtacı tavuklarda diyet probiyotik takviyesinin yumurta ağırlığı üzerinde önemli bir etkisi vardır".

Anahtar Kelimeler: Probiyotik, *Bacillus subtilis*, yumurta ağırlığı, yumurtacı tavuklar, meta-analizi

To cite this article: Kılıç İ. Berk İ. Bozkurt Z. Doğan Y.N. The Effect of Dietary Probiotic Supplementation on Egg Weight in Laying Hens: A Meta–Analysis Study. Kocatepe Vet J. (2020) 13(2):145-151

Submission: 27.02.2020 **Accepted**: 25.04.2020 **Published Online**: 04.05.2020
ORCID ID; İK: 0000-0003-0595-8771, İB: 0000-0002-4008-2480, ZB: 0000-0001-8272-7817, YND: 0000-0002-1309-0936
*Corresponding author e-mail: kilicibrahim@hotmail.com

INTRODUCTION

Due to the long–term genetic selection to improve economically important production characteristics such as meat and eggs, the birds' tolerance for negative environmental conditions decreased while yields increased, animal health and well–being were adversely affected. Probiotics have been used instead of therapeutic agents such as antibiotics to struggle with stress (Fathi et al. 2018) and diseases (Forte et al. 2016a) caused by intensive production methods (Kopp-Hollihan 2001). More commonly, the probiotics, containing beneficial and viable microorganisms that balance the gut microbiota are used as commercial feed additives in poultry feeds to improve growth rate and laying performance by increasing the utilization of feed (Fuller 1989). *Bacillus subtilis* is the most commonly used probiotic species in animal feeding (Simon et al. 2001). It was reported that the addition of *B. subtilis* to the laying hen's feed improve the balance of gut microbiota and intestinal absorption capacity (Abdelqader et al. 2013), enhance immune response (Zhang et al. 2012), decrease the cholesterol content in egg yolk (Sobczak and Kozłowski 2015) and promote bird growth (Lee et al. 2015). Although there are reports that the supplementation of probiotics in poultry feed has positive effects on egg weight, internal and external egg quality traits, these reports conflict with each other (Fathi et al. 2018) and do not demonstrate clearly the beneficial effect of probiotics. In such cases where different results can be found in different studies, meta–analysis provides a more general and excellent solution.

Meta–analysis is a statistical method that helps to combine qualitative and quantitative research results carried out on the same subject in different places, times and centers to reach a general conclusion (Boissel et al. 1989). As it looks at special aspects rather than relies solely on judgment, meta–analysis uses quantitative methods and this differentiates it from the classic reviews in literature (Mosteller and Colditz 1996). According to Yach (1990), meta–analysis is part of the re-examination process. Additionally, it deals with data analysis that draws results from the main study and uses quantitative methods to explain the heterogeneity of the results, calculating the combined overall impact. In brief, meta–analysis is a method of evaluating previous studies (Dawson et al. 1994).

The aim of meta–analysis is to achieve the most accurate quantitative results by combining the studies carried out via small samples and increasing the total sample range. Thus, a more reliable estimation of parameters is ensured and the inconsistencies that emerge in scientific literature can be evaluated. Furthermore, this method, in which the size of the common (overall) effect is determined, turns small–scale omitted reports into effective and useful materials (Fitz-Gibbon 1985, Cohen and Manion 2001).

In the light of this information, the aim of this study was to determine whether the probiotic supplementation (*B. subtilis*) was effective on egg weight in laying hens by meta–analysis.

MATERIALS and METHODS

The following criteria were taken into consideration for eligibility and the studies selection in meta-analysis process, respectively.

1. Genotype 2. Hen age 3. Probiotic type 4. Probiotic dose 5. Database 6. Publication year range.

Within the framework of the study protocol, the limitations of the studies included into meta–analysis conducted in this study are as follows: Brown and white laying hens were in the age range of 18 – 42 – weeks; The probiotic type was *B. subtilis*; the dosage of *B. subtilis* was in the range of 400 – 1000 g/ton (400 – 1000 g per 1 ton feed) as $10^7 – 10^{10}$ CFU/g. In meta-analysis studies, a database should be determined for the selection of the researches (Boissel et al. 1989, Yach 1990, Mosteller and Colditz 1996, Dawson et al. 1994). In this study, the Web of Science was determined as database. Studies have been published in the last 15 years in journals indexed at least SCI–exp. Reason for selecting articles indexed at SCI–exp was to set a certain restriction for search, and it was decided that there would be journals above a certain level of impact factor. The keywords searched according to the study protocol were laying hens, probiotic, *B. Subtilis* and egg weight. In this study, 37 studies were examined and 8 studies (Mahdavi et al. 2005, Xu et al. 2006, Zhang et al. 2012, Amani et al. 2013, Sobczak and Kozłowski 2015, Forte et al. 2016b, Mazanko et al. 2017, Hosseindoust et al. 2018) that met the above conditions were selected and subjected to meta-analysis. In these studies, the difference between experimental and control groups was examined and none of them were statistically significant. In this framework, the hypothesis tested by meta–analysis of the study is presented below: *Hypothesis:* The dietary probiotic supplementation (*B. subtilis*) has a significant effect on egg weight in laying hens.

In the study, there were no a confounding variables whose presence affects the variables being studied (or the confounding effects which may related to the basic hypothesis). This study is a meta-analysis study and it was carried out by calculating the effect size using means calculated for experimental–control groups in continuous data. Hedges g effect sizes for each study were calculated by equation 1, Cohen's d effect sizes by equation 2 and Jacobian correction coefficient *j* by equation 3. Begg rank correlation, Egger regression methods and funnel plot were used to determine bias of studies. To determine the heterogeneity between studies, Cochran's Q statistics (equation 4) were used and the heterogeneity criteria H, T^2 and I^2 were calculated (Cochran 1954, Hedges 1981, Cohen 1988, Begg and Mazumdar 1994, Egger et al. 1997, Higgins and Thompson 2002, Borenstein et al. 2009).

$$g = J * d \qquad (1)$$

$$d = \frac{\bar{X}_1 - \bar{X}_2}{S_{within}} \qquad (2)$$

$$J = 1 - \frac{3}{4(n_1 + n_2 - 2) - 1} \qquad (3)$$

$$Q = \sum_{i=1}^{k} W_i ES_i^{\,2} - \frac{\left(\sum_{i=1}^{k} W_i ES_i\right)^2}{\sum_{i=1}^{k} W_i} \qquad (4)$$

Since the heterogeneity between the studies was determined, the overall effect size was calculated by the random effect model. Because the variance between studies is also taken into account in the random effect model, variance and standard error corrections were made. The data were analyzed by Microsoft Excel and Comprehensive Meta-Analysis Trial Software.

RESULTS and DISCUSSION

In this study which examined the effect of *B. subtilis* on egg weight in laying hens by meta-analysis study, the effect size was calculated for each study and the obtained findings were given in Table 1. According to these results, it has been determined that the study of Sobczak and Kozłowski (2015) the smallest effect size (Hedges g = 0.040), while the study of Ammani et al. (2013) has the biggest effect size (Hedges g = 0.683). The effect sizes of other studies ranged between these two effect sizes.

According to the total values shown in Table 1, Cochran's Q statistics were calculated as 46.635 – (110.879) 2 / 502.216 = 22.155. This value was given in Table 2. The findings for the determination of heterogeneity among the studies were given in Table 2. According to Table 2, the heterogeneity among studies was determined with respect to 3 criteria (in the Cochran Q test, Q = 22.155 > 14.07 and p < 0.05; H = 2.881 – the H statistic does not contain confidence intervals 1–; $T^2 > 0$). Moreover, I^2 statistics showed that there is medium level heterogeneity with 65.285%. On the other hand, the results of Begg's rank correlation method (z = 0.521; p = 0.602) and Egger's regression test (t = 0.149; p = 0.886) showed that there was no publication bias for studies. Also, funnel plot regarding publication bias was given in Figure 1.

As the heterogeneity between studies was determined from the findings in Table 2, the random effect model was used instead of the fixed effect model in the general effect size calculations. In the random effect model, the total variance (V_{ES}) of the studies is the sum of the variance within the studies and the variance between the studies. Weight is $W = 1 / V_{ES}$. Total values used for calculation of the overall effect size in the random effect model were given in Table 3.

The overall effect size using the random effect model was calculated as M = 32.69 / 146.26 = 0.223 from the formula M = Σ(W*ES) / ΣW. The findings of the overall effect size were given in Table 4. Here, the estimated variance and standard error of the overall effect size was found from the formulas $V_M = 1 / \Sigma W$ and $V_M = (SE_M)^2$. According to the results in Table 4, the overall effect size was found as 0.223. Based on z and p values, the hypothesis of the study was accepted (z = 2.90; p < 0.05). In other words, "the probiotic has a significant effect on egg weight in laying hens". This effect can be defined as the "low impact" in the effect size classification of Cohen (1988). Because, Cohen (1988) made classified effect size around 0.20 as "low", 0.50 as "medium" and 0.80 as "high".

There are some studies reporting that probiotics do or do not affect (Panda et al. 2003, Zarei et al. 2011, Sheoran et al. 2018) egg weight (Mahdavi et al. 2005, Kalavathy et al. 2009). A similar inconsistent status is also available for internal and external quality traits of eggs (Zhang et al. 2012, Youssef et al. 2013). While Mahdavi et al. (2005) showed the effect on albumin quality, Xu et al. (2006) proved increasing egg weight. On the other hand, Sobczak and Kozlowski (2015) and Hosseindoust et al. (2018) did not find the results statistically significant. These results show that using probiotics is effective on egg weight and other traits but this could not be clearly demonstrated.

In each of the 8 studies that were included into this study, the number of laying hens used ranged from 20 to 300 and the differences between experimental and control groups were not statistically significant for all studies. So, there were 1154 laying hens in total for each group in the meta-analysis. The average rate of increase in egg weight was about 2% in 8 studies. However, this increase was not statistically significant when considered for a single study because the sample size was not sufficient for statistical significance. In other words, statistical significance is affected by the sample size. This increase ratio (2%) becomes statistically significant as more samples are used, since the studies are combined with meta-analysis.

A meta-analysis diagram of the random effect model was shown in Figure 2. The effect sizes and relative weights of each study, overall effect size and Forest graph were given in Figure 2. The square shape "■" in the forest graph indicates the effect size, the size of the squares of the relative weight of the studies, the width of lines in the 95% confidence interval while diamond shape "♦" indicates the overall effect size of each study. The relative weight is the percentage of the study weight and the highest relative weight (17.97%) was calculated for the study done by Forte et al. (2016b).

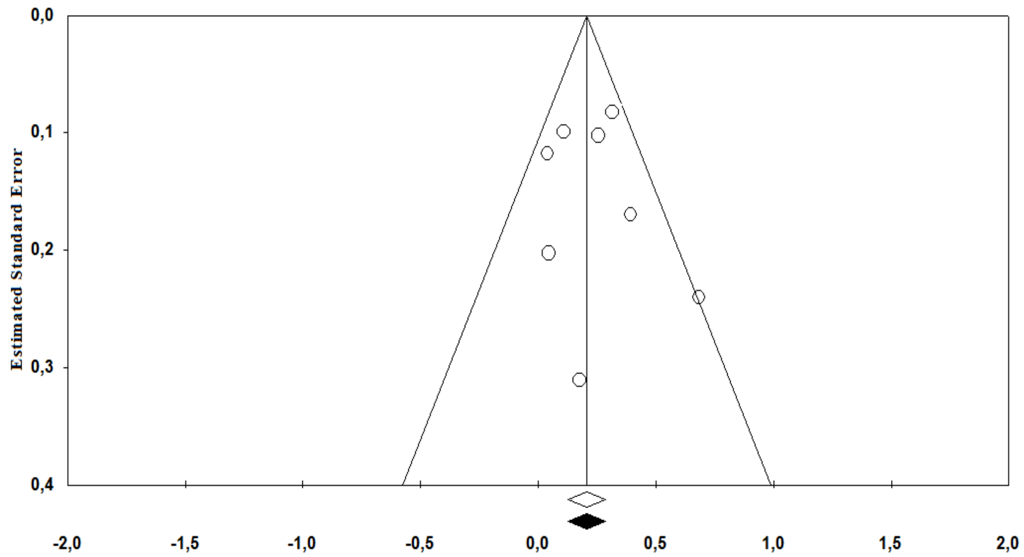
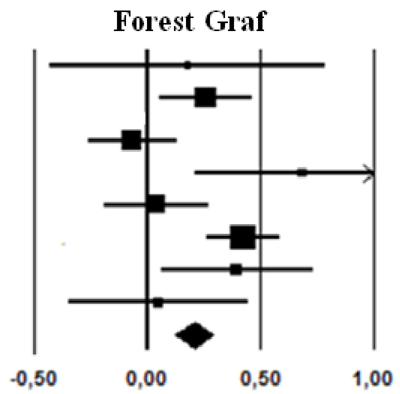


Figure 1. Funnel plot regarding publication bias

Studies	ES	RW %
Mahdavi *et al.*	0.177	5.35
Xu *et al.*	0.256	16.39
Zhang *et al.*	-0.067	16.59
Ammani *et al.*	0.683	7.69
Sobczak and Kozłowski	0.040	15.17
Forte *et al.*	0.420	17.97
Mazanko *et al.*	0.393	11.38
Hosseindoust *et al.*	0.047	9.46
Overall	**0.223**	



RW: Relative weight

Figure 2. A meta–analysis diagram of the random effect model

Table 1. The effect size for each study

Studies	Cohen's d	*J*	Hedges g ES	W	W*ES	W*ES²
Mahdavi et al. (2005)	0.180	0.980	0.177	10.367	1.834	0.324
Xu et al. (2006)	0.256	0.998	0.256	95.593	24.452	6.254
Zhang et al. (2012)	-0.067	0.998	-0.067	100.321	-6.747	0.454
Ammani et al. (2013)	0.690	0.989	0.683	17.359	11.855	8.096
Sobczak and Kozłowski (2015)	0.040	0.997	0.040	72.365	2.880	0.115
Forte et al. (2016a/2016b)	0.421	0.999	0.420	147.123	61.812	25.970
Mazanko et al. (2017)	0.395	0.995	0.393	34.706	13.650	5.368
Hosseindoust et al. (2018)	0.047	0.992	0.047	24.382	1.143	0.054
Total				**502.216**	**110.879**	**46.635**

ES: Effect Size; W: Weight

Table 2. The findings for the determination of heterogeneity or homogeneity among studies

Methods	Parameters	df	Chi square table value	P
Cochran's Q test	22.155	7	14.07	< 0.05
		95% Confidence Interval		
		Min.	Max.	
H	2.881	2.456	3.379	
T^2	0.031	0.018	0.049	
I^2	65.285	52.189	74.795	

Begg's rank correlation z = 0.521; P = 0.602 and Egger's regression test t = 0.149; P = 0.886 for publication bias

Table 3. Total values used for the overall effect size in the random effect model

Studies	Hedges g (ES)	SE_{ES}	V_{ES}	W	W*ES
Mahdavi et al. (2005)	0.177	0.357	0.128	7.830	1.385
Xu et al. (2006)	0.256	0.204	0.042	23.971	6.132
Zhang et al. (2012)	-0.067	0.203	0.041	24.258	-1.632
Ammani et al. (2013)	0.683	0.298	0.089	11.253	7.685
Sobczak and Kozłowski (2015)	0.040	0.212	0.045	22.185	0.883
Forte et al. (2016a/2016b)	0.420	0.195	0.038	26.279	11.041
Mazanko et al. (2017)	0.393	0.245	0.060	16.647	6.547
Hosseindoust et al. (2018)	0.047	0.269	0.072	13.837	0.649
Total				**146.260**	**32.690**

Table 4. The findings of overall effect size

Model	Overall effect size	V_M	SE_M	95% Confidence Interval		z value	P
				Min.	Max.		
Random Effects Model	0.223	0.006	0.077	0.073	0.374	2.90	< 0.05

CONCLUSION

Eight studies were investigated by meta-analysis and the sample size was a total of 1154 laying hens for each group (experimental and control groups) in the study. It was detected that using probiotics had an effect on egg weight and thus, a more general result was obtained. As a matter of fact, it was possible to encounter different findings in the literature. These results emphasized once again the importance of meta-analysis that can provide certain results by combining studies that have been worked with a small sample size. More reliable scientific results can be revealed by extending the time span and combining more studies with meta–analysis because working with larger samples gives a more realistic result.

ACKNOWLEDGEMENT

This article is a summary of the second author's Master thesis.

Conflict of Interest: The authors declare that they have no conflict of interest.

REFERENCES

Abdelqader A, Irshaid R, Al-Fataftah AR. Effects of dietary probiotic inclusion on performance, eggshell quality, cecal microflora composition, and tibia traits of laying hens in the late phase of production. Trop Anim Health Pro. 2013; 45: 1017 – 1024. doi.org/10.1007/s11250-012-0326-7

Amani W, Youssef HMA, Hassan HM, Mohamed MA. Effect of probiotics, prebiotics and organic acids on layer performance and egg quality. Asian J Poult Sci. 2013; 7: 65 – 74. doi.org/10.3923/ajpsaj.2013.65.74

Begg CB, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. Biometrics. 1994; 50:1088-1101.

Boissel JP, Blanchard J, Panak E. Considerations for the Meta – Analysis of randomized clinical trials. Control Clin Trials. 1989; 10: 254 – 281.

Borenstein M, Hedges LV, Higgins JPT, Rothstein HR. Introduction to Meta-Analysis. UK: John Wiley and Sons, Ltd., Publication; 2009.

Cochran WG. The combination of estimates from different experiments. Biometrics. 1954; 10: 101 – 129. doi.org/10.2307/3001666

Cohen J. Statistical Power Analysis for the Behavioral Sciences. New York, USA: Academic Press; 1988.

Cohen L, Manion L. Research Mehhods in Education. New York, USA: Rotledge Falmer; 2001.

Dawson B, Saunders B, Trapp G. Basic and Clinical Biostatistics. ABD: Appleton and Lange, Norwalk; 1994.

Egger M, Smith GD, Phillips AN. Principles and Procedures. BMJ. 1997; 315: 1533 – 1537. doi.org/10.1136/bmj.315.7121.1533

Fathi M, Al-Homida I, Al-Dokhail A, Ebeid T, Abou-Emera O, Alsagan A. Effects of dietary probiotic (*Bacillus subtilis*) supplementation on productive performance, immune response and egg quality characteristics in laying hens under high ambient temperature. Ital J Anim Sci. 2018; 17: 804–814. doi.org/10.1080/1828051X.2018.1425104

Fitz-Gibbon CT. The implications of meta analysis for educational research. Brit Educ Res J. 1985; 11: 45 - 49. doi.org/10.1080/0141192850110105

Forte C, Acuti G, Manuali E, Casagrande Proietti P, Pavone S, Trabalza-Marinucci M, Moscati L, Onofri A, Lorenzetti C, Franciosini MP. Effects of two different probiotics on microflora, morphology, and morphometry of gut in organic laying hens. Poultry Sci. 2016a; 95: 2528–2535. doi.org/10.3382/ps/pew164

Forte C, Moscati L, Acuti G, Mugnai C, Franciosini MP, Costarelli S, Cobellis G, Trabalza-Marinucci M. Effects of dietary *Lactobacillus acidophilus* and *Bacillus subtilis* on laying performance, egg quality, blood biochemistry and immune response of organic laying hens. J Anim Physiol An N. 2016b; 100: 977 – 987. doi.org/10.1111/jpn.12408

Fuller R. Probiotics in man and animals. J Appl Bacteriol. 1989; 66: 365 – 378.

Hedges L. Distribution theory for Glass's estimator of effect size and related estimators. J Educ Stat. 1981; 6: 107 – 128. doi.org/10.3102/10769986006002107

Higgins JPT, Thompson SG. Quantifying heterogeneity in a meta analysis. Stat Med. 2002; 21: 1539 – 1558. doi.org/10.1002/sim.1186

Hosseindoust A, Mohammadi M, Yao ZP, Jung M, Kim IH. Dietary *Bacillus subtilis* B2A strain in laying hens challenged with *Salmonella gallinarum*: effects on egg production, egg quality, blood haptoglobin and targeted intestinal Salmonella shedding. J Appl Anim Res. 2018; 46: 512 – 517. doi.org/10.1080/09712119.2017.1351369

Kalavathy R, Abdullah N, Jalaludin S, Wong M, Ho YW. Effect of Lactobacillus cultures on performance of laying hens and total cholesterol, lipids and fatty acid composition of egg yolk. J Sci Food Agr. 2009; 89: 482 – 486. doi.org/10.1002/jsfa.3477

Kopp-Hollihan L. Prophylactic and therapeutic uses of probiotics: A review. J Am Diet Assoc. 2001; 10: 229–238. doi.org/10.1016/S0002-8223(01)00060-8

Lee KW, Kim DK, Lillehoj HS, Jang SI, Lee SH. Immune modulation by *Bacillus subtilis*-based direct-fed microbials in commercial broiler chickens. Anim Feed Sci Tech. 2015; 200: 76 –85. doi.org/10.1016/j.anifeedsci.2014.12.-006

Mahdavi AH, Rahmani HR, Pourr J. Effect of probiotic supplements on egg quality and laying hen's performance. International Journal of Poultry Science. 2005; 4: 488 – 492.

Mazanko MS, Gorlov IF, Prazdnova EV, Makarenko MS, Usatov AV, Bren AB, Chistyakov VA, Tutelyan AV, Komarova ZB, Mosolova NI, Pilipenko DN, Krotova OE, Struk AN, Lin A, Chikindas ML. Bacillus probiotic supplementations ımprove laying performance, egg quality, hatching of laying hens, and sperm quality of roosters. Probiot Antimicrobiol Proteins. 2017; 10: 367 – 373. doi.org/10.1007/s12602-017-9369-4.

Mosteller F, Colditz GA. Understanding research synthesis (Meta Analysis). Annu Rev Publ Health. 1996; 17:1-23. doi.org/10.1146/annurev.pu.17.050196.000245

Panda AK, Reddy MR, Rama RA, Praharah NK. Production performance, serum/yolk cholesterol and immune competence of white leghorn layers as influenced by dietary supplementation with probiotic. Trop Anim Health Pro. 2003; 35: 85 – 94.

Sheoran N, Maan S, Kumar A, Batra K, Chaudhary D, Sihag S, Kumar V, Maan NS. Probiotic and prebiotic supplementation improving the production performance and immune characteristics of laying hens. Indian J Anim Res. 2018; 52:1433 – 1439. doi.org/10.18805/ijar.B-3394

Simon O, Jadamus A, Vahjen W. Probiotic feed additives—Effectiveness and expected modes of action. J Anim Feed Sci. 2001; 10: 51 – 67. doi.org/10.22358/jafs/70012/2001

Sobczak A, Kozłowski K. The effect of a probiotic preparation containing *Bacillus subtilis* ATCC PTA-6737 on egg production and physiological parameters of laying hens. Ann Anim Sci. 2015; 15: 711 – 723. doi.org/10.1515/aoas-2015-0040

Xu CL, Ji C, Ma Q, Hao K, Jin ZY, Li K. Effects of a dried *Bacillus subtilis* culture on egg quality. Poultry Sci. 2006; 85: 364 – 368. doi.org/10.1093/ps/85. 2.364.

Yach D. Meta-analysis in epidemiology. S Afr Med J. 1990; 78: 94 – 97.

Youssef AW, Hassan IM, Ali IM, Mohamed MA. Effect of probiotics, prebiotic and organic acids on layer performance and egg quality. Asian J Poult Sci. 2013; 7: 65 – 74. doi.org/10.3923/ajpsaj.2013.65.74

Zarei M, Ehsani M, Torki M. Dietary inclusion of probiotics, probiotics and symbiotic and evaluating performance of laying hens. Am J Agric Biol Sci. 2011; 6: 249 – 255.

Zhang JL, Xie QM, Ji J, Yang WH, Wu YB, Li C, Ma JY, Bi YZ. Different combinations of probiotics improve the production performance, egg quality, and immune response of layer hens. Poultry Sci. 2012; 91: 2755 – 2760. doi.org/10.3382/ps.2012-02339.