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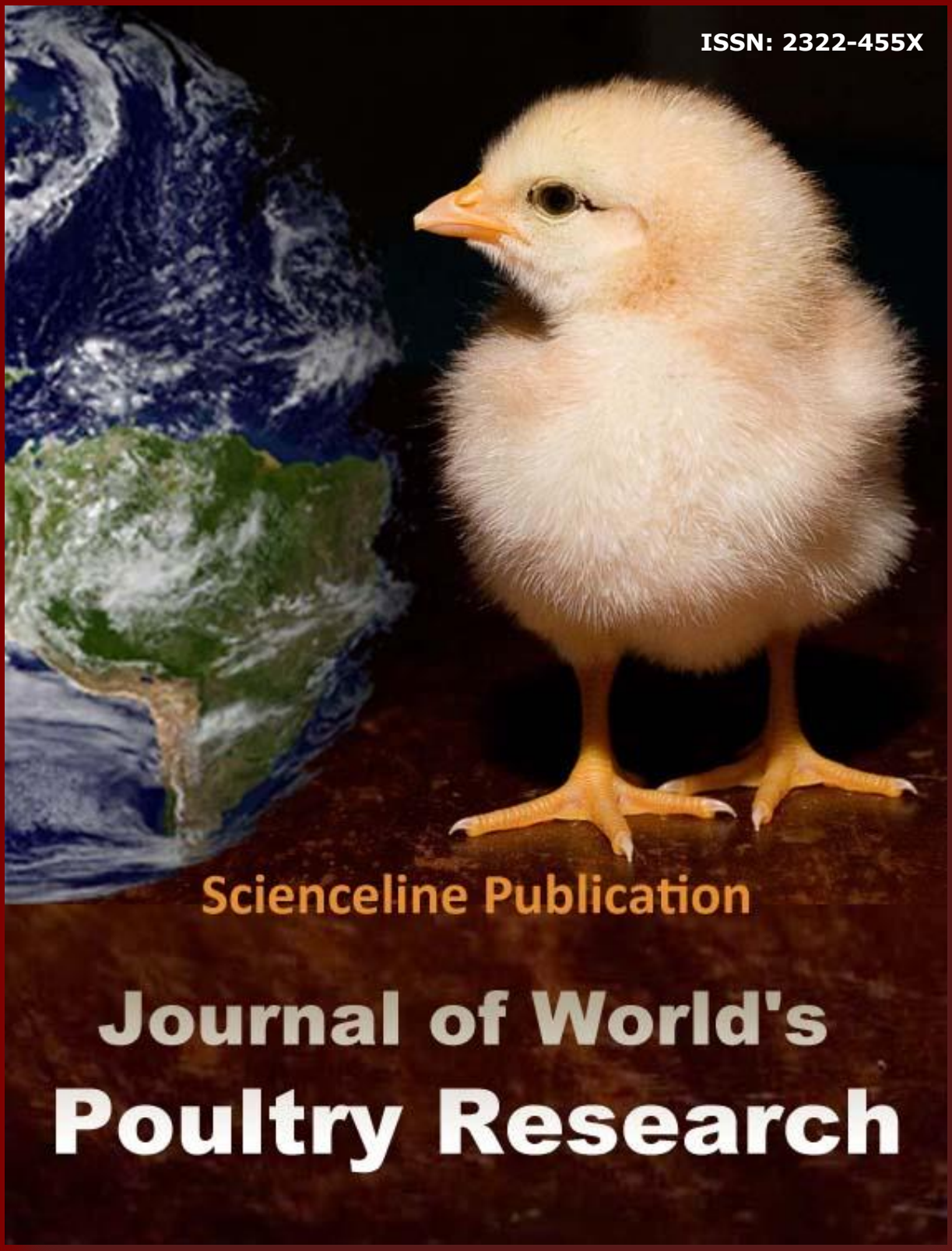

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Journal of World's Poultry Research

J. World Poult. Res. 9 (3): 117-159; September 25, 2019

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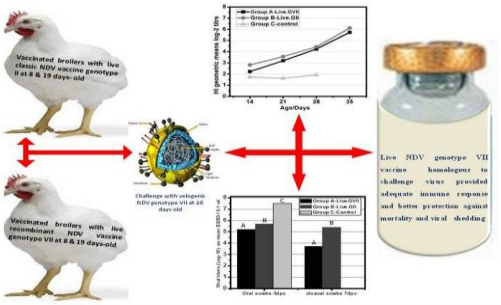
Volume 9 (3); September 25, 2019

Research Paper

Advancement in Vaccination of Broiler Chickens with Genotype-Matched Vaccines to Currently Epidemic Newcastle Disease Virus Genotype VII in Egypt.

Amer SAM, Ali MA, Kandeil AM and Kutkat MA.
J. World Poult. Res. 9(3): 117-123, 2019; pii: S2322455X1900015-9
DOI: https://dx.doi.org/10.36380/jwpr.2019.14



To cite this paper: Amer SAM, Ali MA, Kandeil AM and Kutkat MA (2019). **Advancement in Vaccination of Broiler Chickens with Genotype-Matched Vaccines to Currently Epidemic Newcastle Disease Virus Genotype VII in Egypt.** *J. World Poult. Res.*, 9 (3): 117-123.

ABSTRACT: Newcastle disease virus (NDV) outbreaks still occur frequently in Egypt in spite of the heavy implementation of classic NDV vaccines for a long time ago, where NDV genotype VII has become the dominant genotype in Egypt from 2012 until now. Many previous studies have recommended using genotype-matched NDV vaccines against the epidemic virus for providing better protection and minimizing virus shedding. Therefore, the present study evaluated the efficacy of two available live NDV vaccines in Cobb 500 broilers. The group A and B (20 birds each) were vaccinated with live attenuated NDV vaccines genotype VII and II, respectively with double doses at 5 and 19 days of age. Also, group C consisting of 20 unvaccinated birds was studied as a control group. The efficacy of live vaccines was determined using virus challenge test. Hence, all groups were challenged with velogenic NDV genotype VIId at a dose equivalent to $10^{6.0}$ 50 percent Embryo Infective Dose (EID_{50}) via the intramuscular route at 28 days-old. Serum antibodies level was assessed by hemagglutination inhibition test. Moreover, virus shedding was measured by EID_{50}. The obtained results indicated that vaccinated birds had similar haemagglutination titers with no significant difference prior challenge. Meanwhile, group A showed significant protection against mortality, as well as a significant reduction in virus shedding 7 days post-challenge compared to Group B. We concluded that live recombinant-genotype VII vaccine homologous to challenge virus could improve the protective efficiency in chicken against NDV compared to live classic genotype II vaccine. It is suggested that the implementation of genotype-matched NDV vaccines confer better protection in commercial broilers vaccination programs.
Keywords: Broilers, Genotype-matched vaccine, Genotype VII, Newcastle disease virus

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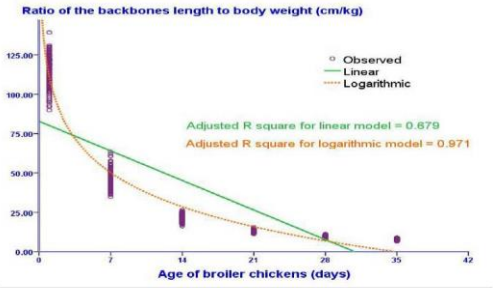
Research Paper

Model Estimation for Longitudinal Bone Growth Based on Age in Male and Female Commercial Broiler Chickens.

Kongpechr S, Sohsuebngarm D and Sukon P.
J. World Poult. Res. 9(3): 124-132, 2019; pii: S2322455X1900016-9
DOI: https://dx.doi.org/10.36380/jwpr.2019.15



Citation: Kongpechr S, Sohsuebngarm D and Sukon P (2019). **Model Estimation for Longitudinal Bone Growth Based on Age in Male and Female Commercial Broiler Chickens.** *J. World Poult. Res.*, 9 (3):124-132.

ABSTRACT: Longitudinal bone growth is essential to support rapid body growth in commercial broiler chickens. The present study aimed to determine which simple mathematic model is best suitable for explaining the absolute and the relative bone growth in length (expressed as a ratio of bone length to body weight) as a function of age in male and female commercial broiler chickens over the first 35 days of age. A total of 1,800 broiler chickens (900 males and 900 females) of Cobb 500, Ross 308, and Arbor Acres raised in standard commercial broiler houses were randomly selected for this study. Body weight and the lengths of backbones, third toe, shank bone, and keel bone were individually measured in all chickens at 1, 7, 14, 21, 28, and 35 days of age. Regression analysis (with 4 simple mathematical models including linear, logarithmic, inverse, and exponential) was used to find a suitable model for estimating the absolute and the relative bone growth in length. In addition, an adjusted R2 was used to assess the model fitting. The results indicated that the absolute bone growth in length linearly increased with age. The adjusted R2 values for the linear model were 0.973, 0.937, 0.950, and 0.974 for the lengths of the backbones, third toe, shank bone, and keel bone, respectively. However, the relative bone growth in length logarithmically decreased with age. The adjusted R2 values of the logarithmic model were 0.971, 0.952, 0.957, and 0.905 for the relative length of the backbones, third toe, shank bone, and keel bone, respectively.The present investigation suggests thata linear model is a suitable model for estimating the absolute bone growth in length, but a logarithmic model is a proper model for estimating the relative bone growth in length of commercial broiler chickens.
Keywords: Age, Bone growth, Broiler chickens, Mathematical model, Regression

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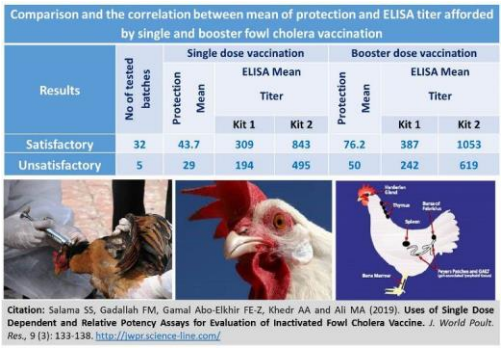
Research Paper

Uses of Single Dose Dependent and Relative Potency Assays for Evaluation of Inactivated Fowl Cholera Vaccine.

Salama SS, Gadallah FM, Gamal Abo-Elkhir FE-Z, Khedr AA and Ali MA.
J. World Poult. Res. 9(3): 133-138, 2019; pii: S2322455X1900017-9
DOI: https://dx.doi.org/10.36380/jwpr.2019.16


Comparison and the correlation between mean of protection and ELISA titer afforded by single and booster fowl cholera vaccination

Results	No of tested batches	Single dose vaccination			Booster dose vaccination		
		Protection	Mean ELISA Titer		Protection	Mean ELISA Titer	
			Kit 1	Kit 2		Kit 1	Kit 2
Satisfactory	32	43.7	309	843	76.2	387	1053
Unsatisfactory	5	29	194	495	50	242	619

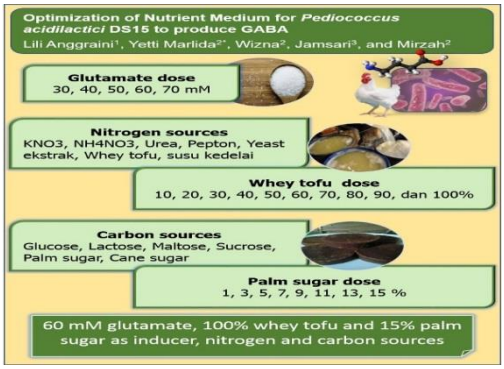
ABSTRACT: Fowl cholera is a septicemic respiratory complex caused by *Pasteurella multocida*, widely distributed in poultry and other avian species and of major economic importance. A total of 37 different inactivated *Pasteurella multocida* vaccines from different sources either locally prepared or imported from different sources were comparatively tested for relative potency following both single dose and booster dose vaccination assays. The study objective was to minimize the time factor exhausted in the evaluation processes of the inactivated fowl cholera vaccines. So it is planned to compare between single and booster dose vaccinations and their related potency. Correlation between protection associated with the single dose and booster dose vaccination were evaluated and average requirement for protection was 43.7% in single dose vaccination assay compared to 76.2 % associated with booster dose vaccination assay. In the same concern, the correlation between both assays for the seroconversion was estimated using ELISA and the minimum requirement was 1.8× cut off value in the single dose vaccination assay compared to 2.25× cut off value in the booster dose vaccination assay. In conclusion, single dose vaccination assay could be valuable in the evaluation of inactivated fowl cholera vaccines through determination of protection indices and/or estimation of humoral immune response if the above mentioned data is considered.
Keywords: Challenge and chicken, ELISA, Inactivated vaccine, *Pasteurella multocida*, Single dose

[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Optimization of Nutrient Medium for *Pediococcus acidilactici* DS15 to Produce GABA.

Anggraini L, Marlida Y, Wizna W, Jamsari J and Mirzah M.
J. World Poult. Res. 9(3): 139-146, 2019; pii: S2322455X1900018-9
DOI: https://dx.doi.org/10.36380/jwpr.2019.17

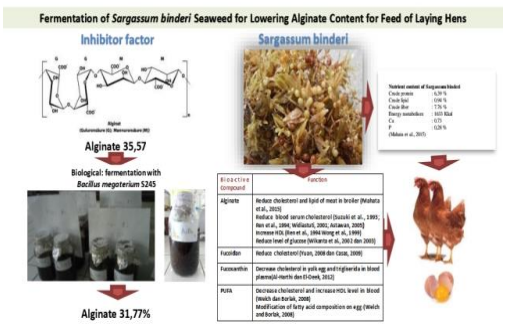


ABSTRACT: Nutrition is an essential factor for microorganisms to grow and survive. Carbon and nitrogen sources are used in producing primary and secondary metabolites. Gamma-Aminobutyric acid (GABA), a non-coded amino acid, is a secondary metabolite which acts as an inhibitory neurotransmitter of the central nervous system. *Pediococcus acidilactici* DS15 is a bacterium belonging to the order of Lactic Acid Bacteria. This study aimed to determine the effects of nutrients including glutamate, nitrogen and carbon sources on GABA production by *Pediococcus acidilactici* DS15. The tests were carried out using a range of 30 mM, 40 mM, 50 mM, 60 mM, and 70 mM glutamate as inducer and carbon sources in the form of peptone, yeast extract, skim milk, NH_4NO_3, KNO_3, whey tofu and soy milk as nitrogen sources, and then glucose, lactose, maltosa, sucrose, palm, and cane sugar as carbon sources. The best sources of both will be tested with levels 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% for nitrogen and 1, 3, 5, 7, 9, 11, 13 and 15% for carbon. The results of this investigation revealed that the addition of 60 mM glutamate caused the higher amount of GABA production and the best source of nitrogen and carbon for *Pediococcus acidilactici* DS15 were 100% whey tofu and 15% palm sugar, respectively. Production rate of GABA by *Pediococcus acidilactici* DS15 could reach up to 311,485 mg / L.
Keywords: Carbon, GABA, Glutamate, Nitrogen, *Pediococcus acidilactici* DS15
[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Fermentation of Sargassum binderi Seaweed for Lowering Alginate Content of Feed in Laying Hens.

Dewi YL, Yuniza A, Sayuti K, Nuraini and Mahata ME.
J. World Poult. Res. 9(3): 147-153, 2019; pii: S2322455X1900019-9
DOI: https://dx.doi.org/10.36380/jwpr.2019.18



ABSTRACT: The object of this study was to reduce the alginate content of *Sargassum binderi* by fermentation of *Bacillus megaterium* S245 in feed of laying hens. The experiment was arranged in a factorial with completely randomized design. The first factor was different inoculum dosage (1, 3, 5, 7, and 9%), while the second factor was different fermentation period (1, 3, 5, 7 and 9 days), and each treatment was replicated for five times. The measurements were total dry matter, organic matter, ash, alginate, and crude protein. The results showed there were no significant effect of inoculum dosage, and interaction between inoculum dosage and fermentation period on

alginate, total dry matter, organic matter, ash and crude protein content of *Sargassum binderi* while fermentation period reduced the alginate and total dry matter content. Fermentation period also increased the crude protein content significantly. Besides that, fermentation period didn't effect on organic matter and ash content significantly. The fermentation of *Sargassum binderi* with *Bacillus megaterium* S245 at inoculum dosage of 1% and fermentation period of nine days was the best combination for lowering alginate content in *Sargassum binderi* and this treatment had positive effect on nutrient content of *Sargassum binderi*.

Keywords: Alginate, *Bacillus megaterium S245,* Fermentation, Laying hens, *Sargassum binderi*

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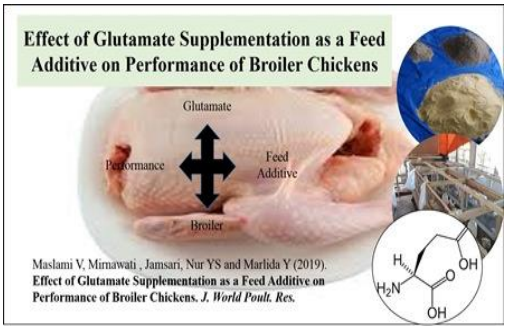
Research Paper



Effect of Glutamate Supplementation as a Feed Additive on Performance of Broiler Chickens.

Maslami V, Mirnawati, Jamsari, Nur YSh and Marlida Y.

J. World Poult. Res. 9(3): 154-159, 2019; pii: S2322455X1900020-9
DOI: https://dx.doi.org/10.36380/jwpr.2019.19

ABSTRACT: Feed additives are ingredients that are added to the ration as growth promoters and enhancement of the immune system. Glutamate is a feed additive that improves performance by improving the quality of the small intestine and enhancing the immune system. The purpose of this study was to know the effect of including glutamate as a feed additive in improving broiler performance. The material used in this study was broiler strain MB 202 from PT Charoen Phokphand Indonesia as many as 240 birds. The design used was a Completely Randomized Design (CRD) trial design, with six treatments and four replications, so that there were 24 cage plots as experimental units. Each experimental unit consisted of 10 chickens. The Glutamate doses in groups were, A (0.4% commercial glutamate; B (0% glutamate); C (0.2% glutamate); D (0.4% glutamate); E (0.6% glutamate); F (0.8% glutamate). The results indicated that glutamate up to 0.8% had significant effects on feed intake body weight gain and feed conversion ratio (1.70%), but it did not affect the percentage of carcass. It is concluded that including the 0.8% glutamate in broiler disets can improve broiler performance with 35 days of maintenance.

Keywords: Feed additive, Glutamate, Growth promoters, Immune system, Performance

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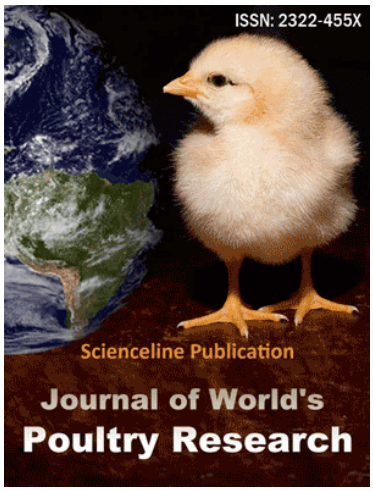

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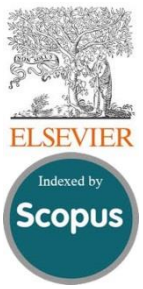

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Advancement in Vaccination of Broiler Chickens with Genotype-Matched Vaccines to Currently Epidemic Newcastle Disease Virus Genotype VII in Egypt

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ABSTRACT

Newcastle disease virus (NDV) outbreaks still occur frequently in Egypt in spite of the heavy implementation of classic NDV vaccines for a long time ago, where NDV genotype VII has become the dominant genotype in Egypt from 2012 until now. Many previous studies have recommended using genotype-matched NDV vaccines against the epidemic virus for providing better protection and minimizing virus shedding. Therefore, the present study evaluated the efficacy of two available live NDV vaccines in Cobb 500 broilers. The group A and B (20 birds each) were vaccinated with live attenuated NDV vaccines genotype VII and II, respectively with double doses at 5 and 19 days of age. Also, group C consisting of 20 unvaccinated birds was studied as a control group. The efficacy of live vaccines was determined using virus challenge test. Hence, all groups were challenged with velogenic NDV genotype VIId at a dose equivalent to $10^{6.0}$ 50 percent Embryo Infective Dose (EID_{50}) via the intramuscular route at 28 days-old. Serum antibodies level was assessed by hemagglutination inhibition test. Moreover, virus shedding was measured by EID_{50}. The obtained results indicated that vaccinated birds had similar haemagglutination titers with no significant difference prior challenge. Meanwhile, group A showed significant protection against mortality, as well as a significant reduction in virus shedding 7 days post-challenge compared to Group B. We concluded that live recombinant-genotype VII vaccine homologous to challenge virus could improve the protective efficiency in chicken against NDV compared to live classic genotype II vaccine. It is suggested that the implementation of genotype-matched NDV vaccines confer better protection in commercial broilers vaccination programs.

Key words: Broilers, Genotype-matched vaccine, Genotype VII, Newcastle disease virus

INTRODUCTION

Newcastle Disease (ND) is a highly contagious viral infection of avian species, including domestic poultry. The causative agent of ND is known as avian paramyxovirus 1 or ND Virus (NDV) which is an enveloped, single-stranded, non-segmented and negative-sense RNA virus. The NDV is a member of *Avulavirus* genus within the Paramyxovirinae subfamily of Paramyxoviridae family in the Mononegavirales order (Mayo, 2002). Within the single serotype, NDV strains are divided into two classes. Viruses from class I comprise only one genotype and mainly isolated from waterfowl, shorebirds, and wild birds. Class II viruses are found in both wild and domestic avian species and are subdivided into 18 genotypes based on fusion protein gene sequence and phylogenetic analysis (Kim et al., 2007; Miller et al., 2010).

A major shift in the types of NDV strains caused by genotype VII of class II virus which has been identified as prevalent in poultry since it was first detected in the 2000s in China (Liu et al., 2003; Zhang et al., 2010) with severe outbreaks in Europe, Africa, the Middle East, South America and Asia (Lomniczi et al., 1998; Abolnik et al., 2004). Genotype VII of NDV was first identified in Egypt by Radwan et al. (2013), and later, presence of other Egyptian NDV genotype VII isolates, as well as concurrent outbreaks, were reported successfully by several studies (Hussein et al., 2014; Abdel -Aziz et al., 2016; Ewies et al., 2017).

Live-attenuated NDV vaccines are generally used in the first weeks of life in broilers, layers, and breeders, by either systemic or the respiratory route, in order to reduce the risk of infection with virulent strains. Furthermore, live vaccines administered by eye drop or orally have been

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found to induce protective mucosal immunity mediated by Immunoglobulin (Ig) A and provide potent systemic immunity alongside their excellent safety profile (Miller and Koch, 2013). Thus, a highly immunogenic and safe live vaccine can provide a chance to enhance herd immunity through a massive vaccination program by the respiratory route (Al-Garib et al., 2003).

Application of an intensive vaccination programs with annual use of ND vaccines has increased, but ND outbreaks have still occurred periodically across the world with elevated economic losses, mainly resulting from higher mortalities and decreased egg production rates; even in well-vaccinated farms have raised questions about the protective efficacy of conventional vaccines and the antigenic variation of NDV (Cho et al., 2008). Nowadays, LaSota and B1 NDV strains used to produce ND vaccines, have phylogenetically the same genotype of viruses isolated in the 1940s, but are genotypically different or dis-matched from strains causing the recent outbreaks of ND, so as to, recent studies were carried out on the role of genotype-matched vaccines in the control of NDV epidemics which found to provide better protection and reduce virus shedding from infected birds against challenge with virulent genotype VII NDV (Hu et al., 2011; Yang et al., 2016). Therefore, the goal of this study was to explore the effects of the matching genotype of live NDV vaccine strain to the likely virulent challenge virus in commercial broiler chickens on the level of protection and virus shedding.

MATERIALS AND METHODS

Ethical approval

The animal experiments were carried out in strict accordance with and adherence to the relevant policies regarding animal handling as mandated under international, national, and /or institutional guidelines for the care of animals and were approved by the Research Ethical Committee at the National Research Centre, Cairo, Egypt.

Viruses and vaccines

The virus used for the challenge purpose was characterized by sequencing as velogenic NDV (vNDV) genotype VIId designated as "NDV/Chicken/EG-MN/NRC/2015" with a Genbank accession number of (MF418020.1). The virus was propagated in nine-day-old specific pathogen-free embryonated chicken eggs via allantoic cavity inoculation. The virus challenge dose, equal to 6 Log_{10} embryo infective dose (EID_{50}) per 0.5 ml**,** was administered intramuscularly to chickens (OIE, 2012).

Live attenuated NDV vaccines: freeze-dried vaccines containing live attenuated NDV genotype VII (KBNP-C4152R2L strain, Himmvac® Dalguban N Plus 2000 doses) and genotype II (LaSota strain, Jovac ND LaSota ® 1000 doses) were supplied by local agencies. The vaccine doses, equal to 6 Log_{10} EID_{50} in 20 µl per bird, were administered according to the manufacturer's recommendations.

Serology

Blood serum was collected pre and post-challenge (at 14, 21, 28 and 35 days of age) from all birds and evaluated by Hemagglutination Inhibition (HI) assay. The HI assay was performed using inactivated NDV antigen (LaSota strain) according to standard procedures with four haemagglutinating units of virus/ antigen in 0.050 ml (OIE, 2012).

Virus shedding

Shedding of the virus was determined by collecting oropharyngeal and cloacal swabs at 3 and 7 days post-challenge (dpc), respectively. Swabs were collected in 1.5 mL of phosphate buffer saline supplemented with a final concentration of gentamicin (200 µg/mL), penicillin G (2000 units/mL) and amphotericin B (4 µg/mL). The presence of the virus was determined by inoculating clarified swab samples into nine-days-old embryonated specific-pathogen-free chicken eggs and conducting HA assay three days later. Pools of swabs (n = 3 per group) from the same group were clarified via centrifugation at 1000 × g for 15 minutes. Virus titers were calculated by using the standard methods described by (OIE, 2012) and were reported as mean EID_{50} / 0.1 ml on a Log_{10} scale.

Chicken experiments

Sixty one-day-old commercial broiler chicks (Cobb 500®) were provided by the certified local hatchery, divided into three experimental groups (A, B and C) of 20 birds each and reared in separate units with strict biosecurity level. Conventional animal welfare regulations and feed standards were taken into account. Chickens in group A vaccinated with live attenuated NDV vaccine (genotype VIId strain) on 5 and 19 days of age via the oculonasal route. Meanwhile, group B received live attenuated NDV vaccine genotype II (LaSota strain) on day 5 and 19 of age through the oculonasal route. In addition, birds in group C, as a control group, did not

receive vaccines. The three groups were challenged with vNDV genotype VIId on 28 days of age (Table 1).

Table 1. NDV vaccination and challenge schedule in Cobb 500 broiler chickens

Group	Number of birds	Vaccination regimen		Challenge time (day of age) [3]
		Type	time (day of age)	
A	20	Live. GVII[1]	5 & 19	28
B	20	Live.GII[2]	5 & 19	28
C	20	None	None	28

[1] Live attenuated NDV genotype VII vaccine via oculonasal route; [2] Live attenuated NDV genotype II vaccine via oculonasal route; [3] Challenge with velogenic NDV (genotype VIId) via intramuscular route.

Statistical analysis

Data were analyzed by one-way ANOVA with Tukey's post hoc test performed using SPSS version 21 software (SPSS Inc., USA) to determine the significance of differences between treatment and control groups. A p-value ≤ 0.05 was considered statically significant.

RESULTS

Protective efficacy of live NDV vaccines in broilers

Non-vaccinated infected broilers displayed marked depression with severe respiratory signs and greenish diarrhea from 3 to 4 dpc and with a mortality rate of 100% at 4 and 5 dpc. In contrast, the vaccinated birds in group A and B revealed weaker or much less clinical signs (including reduced activity, depression with mild to moderate respiratory manifestations) compared to unvaccinated controls. As well as, vaccinated chickens exhibited varying degrees of protection with significantly lower mortality rates started at 4 dpc in comparison with the non-vaccinated group. In general, group A conferred significant protection against mortality with 25% mortality rate (five out of 20 chickens) at 5 and 6 dpc, whilst mortality rate in group B was 60% (12 out of 20 birds) at 5, 6 and 7 dpc, as shown in table 2.

Table 2. The mortality rate in Cobb 500 broiler chickens 7 days post-challenge with vNDV (Genotype VII)

Group	Number of birds	Challenge time (day of age)	The mortality rate at 7 days Post-challenge	
			Number	%
A [a,b]	20	28	5	25
B [b]	20	28	12	60
C	20	28	20	100

[a] significant difference from group B (P<0.05); [b] significant difference compared to control group C (P<0.05).

In necropsy examination, hemorrhagic spots and/or petechial hemorrhages were found in proventricular glands and also ulceration of cecal tonsils with splenomegaly and severe tracheitis were observed in non-vaccinated infected controls. While, similar, mild or even no gross lesions were observed in vaccinated challenged groups.

Serology

Vaccinated groups A and B exhibited positive HI titers for NDV, which increased throughout the vaccination course with significant higher titers from control group C at all designated tested days. For groups A and B, the antibody titers produced respectively by live NDV genotype VII vaccine or live NDV genotype II vaccine were comparable to each other and not significantly different ranging from 2.22 to 5.73 Vs 2.81 to 6.11, respectively pre- and post-challenge. Moreover, HI titers were low in non-vaccinated infected controls ranging from 1.73 to 1.91 up to challenge day. The data was shown in Table 3.

Table 3. Serology pre and post-challenge with vNDV strain (Genotype VII)

Group	Number of birds	Challenge time (day of age)	HI titer means Log-2 (days of age; N = 8)			
			14	21	28	35
A *	20	28	2.22	3.11	4.23	5.73
B *	20	28	2.81	3.88	4.82	6.11
C	20	28	1.73▸	1.61▸	1.91▸	NT

*Significant difference compared to control group C (P<0.05). ▸HI titre ≤ 2 Log-$_2$ considered negative (OIE, 2012). N : Number of tested samples. NT: Not tested (All Birds of this group died at 4 and 5 days post-challenge).

Virus shedding

All of the oropharyngeal swabs from control and vaccinated birds were positive with clearly detectable titers, although it was significantly reduced in both groups A and B compared to control group C at 3 dpc. Meanwhile, no significant difference was detected in oral shedding at 3dpc between group A and B (5.2 VS 5.7 Log $_{10}$ EID_{50} / 0.1 ml). While, cloacal shedding was significantly lower in group A in comparison to group B (3.7 VS 5.4 Log_{10} EID_{50} / 0.1 ml), at 7 dpc, as shown in table 4.

Table 4. Viral shedding oropharyngeal and cloacal swabs collected from Cobb 500 broiler chickens at 3 and 7 days post-challenge, respectively with vNDV (Genotype VII)

Group	Number of birds	Challenge time (day of age)	Virus Shedding *	
			3 dpc (oropharyngeal)	7 dpc (Cloacal)
A [a,b]	20	28	5.2 [a]	3.7 [b]
B [b]	20	28	5.7 [a]	5.4
C	20	28	7.5	NT

*Viral titers (log-10) expressed as mean embryo infectious doses per 0.1 ml from a pool of oral or cloacal swabs (n=3 per group). [a]Significant difference from control group C at 3dpc (P<0.05). [b]Significant difference from group B at 7dpc (P<0.05). NT: None tested (All Birds of this group died at 4 and 5 days post -challenge). dpc: days post-challenge.

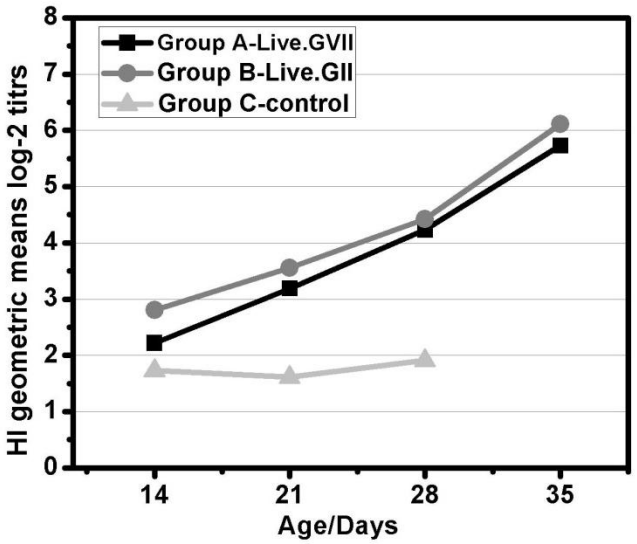

Figure 1. Antibody dynamics of vaccinated and unvaccinated Cobb 500 broiler chickens. Geometric mean Haemagglutination titers (log-2) for sera collected on 14, 21, 28 and 35 days of age (pre-and post-challenge).

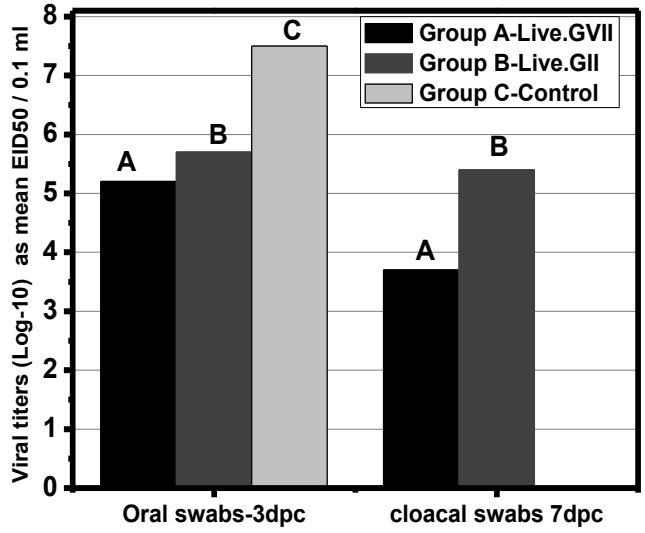

Figure 2. Viral shedding of oral swabs at 3dpc and cloacal swabs at 7dpc in Cobb 500 broiler chickens unvaccinated or vaccinated with live attenuated genotypes (VII or II) NDV vaccines and challenged with vNDV (genotype VIId) expressed as (log-10) mean EID_{50} / 0.1 ml.

DISCUSSION

Despite extensive vaccination regimen, waves of ND outbreaks continue to cause mortality and severe economic losses in poultry flocks (Shahar et al., 2018). The virus that was used in the present challenge was isolated from Egyptian poultry flocks that were heavily vaccinated thus raising the importance of eliminating NDV infection of the flocks.

In spite of all NDV isolates are considered to be of one serotype, vaccination with any NDV strain couldn't provide equal protection against all isolates. Therefore, in fully controlled vaccination experiments it was found that vaccination with the live attenuated vaccine strains was not sufficient to provide protection for birds against challenge with virulent field isolates (Kapczynski and King, 2005). However, vaccines composed of strains more homologous to the challenge virus are more efficient at decreasing morbidities, mortalities, and virus shedding (Miller et al., 2007; Cho et al., 2008; Kim et al., 2017). In the present study, levels of protection induced by the live recombinant genotype VII and live genotype II LaSota NDV vaccines were compared following challenge with the recently acquired genotype VII NDV isolate in commercial broiler chickens.

In birds vaccinated with live vaccines against NDV, subsequently, Immunity develops very early and neutralizing antibodies can be detected 6-10 days after vaccination (Al-Garib et al., 2003; Kapczynski et al., 2013). Furthermore, it is expected that the protection rate reaches a peak near 3 weeks after the initial vaccination and then steadily starts to decrease (Vrdoljak et al., 2017). In the current study, vaccinated groups received the first dose of NDV live vaccine on five days-old to avoid virus neutralization by maternally derived antibodies which are decreased by half every 3-4 days (Umino et al., 1987), and the second dose was administered two weeks later, with the aim to test whether the protection rate will be maintained until the end of the production period. Consequently, the challenge virus was inoculated at 28 days of age, which is the case of birds vaccinated two times two weeks interval and challenged after 23 days from the first dose. Although chickens will not be infected in the field through injection, so far, the intramuscular injection is the recommended route according to (Alexander and Senne, 2008; OIE, 2012) and also for this reasons the eye-drop route did not allow for exacting delivery of infective virus to each bird.

Clinical signs in the challenged birds included usual symptoms related to infection with vNDV such as ruffled feathers, depression, tremor, diarrhea and paralysis which in most cases led to fatality, although a number of vaccinated birds showed much less signs of mild nasal discharge and depression and were found to recover fully by the end of the observation period, especially in group A (genotype VII) that was apparently more protected against clinical disease compared to group B (live LaSota vaccine) as

previously mentioned by (Vrdoljak et al., 2018; Shahar et al., 2018).

In addition to clinical signs, gross post-mortem changes were apparently detected, since in poultry commonly seen as hemorrhages in the spleen, tracheal, proventriculus and cecal tonsils. As well as, the spleen may be enlarged, mottled and necrotic (Susta et al., 2011; Miller and Koch, 2013) which were observed almost in susceptible non-vaccinated flocks which were in agreement with the obtained findings in the present study.

Almost all genotype VII NDV isolates are velogenic strains and resulting in higher mortality rates in poultry reached 100 % (Zhang et al., 2010). Accordingly, trials of vaccination against genotype VII NDV challenge have been carried out by (Dortmans et al., 2014; Susta et al., 2014) concluded that adequate application of live attenuated or inactivated NDV vaccines provided sufficient protection in chickens challenged with vNDV. Consequently, in the current study, the live genotype VII NDV vaccine was introduced into the current commercial broiler vaccination program and its efficacy against vNDV challenge was assessed. Evaluation of the vaccination program showed that Cobb 500 broilers treated with live genotype II vaccines (LaSota) on the day 5 and 19 days of age were more susceptible to vNDV challenge than live NDV vaccine genotype VII at the same designated days-old. These results confirmed that introduction of the recombinant genotype VII NDV live vaccine provided significant protection against mortality compared to live genotype II vaccines (LaSota) at 7dpc, and are consistent with previous investigations by (Cho et al., 2008; Roohani et al., 2015).

Titers of anti-NDV antibodies were measured at second week (post-initial vaccinal dose), one-week prior challenge and also one-week post-challenge. Both live attenuated genotype VII and II NDV vaccines induced a significant immune response compared to non-vaccinated controls. While no significant difference was detected between the vaccinated groups of A and B. However, group B showed slightly higher HI titers than genotype VII in group A which may be due to LaSota vaccine used as mentioned by Miller et al. (2007). The aforementioned study detected higher HI titers when the antigen used in the assay was homologous to the vaccine antigen. Generally, it should be emphasized that the level of antibody titers produced by NDV is not ever the optimum estimate of protection against virus challenge. Correlation between serum anti-NDV titer and protection against NDV challenge is usually more reliable in birds vaccinated with inactivated vaccines because the major immunity induced by killed viruses is a humoral response (Goddard et al., 1988; Reynolds and Maraqa, 2000). In birds vaccinated with live attenuated vaccines, cellular and local immunity contribute considerably to the protection rate by decreasing disease and transmission potential (Kapczynski et al., 2013). In the study by Vrdoljak et al. (2017) have been found that in spite of little or no serum antibody response detected in broilers vaccinated with live attenuated NDV vaccine, birds still showed an almost high level of protection against virus challenge, probably as a result of non-humeral and innate immunity. Thus, the often-used detection of antibody titers in the evaluation of flock`s protection against NDV after vaccination with live attenuated vaccines may under-estimate the actual protection rate (Vrdoljak et al., 2018).

HI antibody titer is one of the most direct factors to estimate the protection induced by ND vaccines, as it corresponds with protection level. More commonly, HI titers of 6 Log-2 or higher are what typically thought of being protective (Raghul et al., 2006). The obtained results in the current investigation emphasized this finding and further revealed that even mortalities and virus shedding following challenge with vNDV were not completely inhibited when HI titers of both live NDV vaccines genotype VII and II were 4.23 and 4.82, respectively at challenge day. Although, live genotype VII vaccine provided significant protection against mortality and viral shedding in compared to live genotype II vaccine at 7 dpc. This finding confirmed that the genotype VII vaccine could reasonably be expected to be effective against vNDV genotype VII infection than genotype II vaccine especially when HI titers are below the protective levels.

Currently, the most widely used vaccines that belong to genotype II such as LaSota provides protection against morbidity and mortality caused by a virulent NDV. Nonetheless, not fully prevents infection or virus shedding in vaccinated birds (Cho et al., 2008). Several previous studies have demonstrated that genotype-matched vaccinations reduce virus shedding following challenge with vNDV isolates more efficiently in comparison to the LaSota strain (Cho et al., 2008; Hu et al., 2009; Miller et al., 2009; Roohani et al., 2015). Similar findings were obtained in the current study in which the live genotype VII vaccine was found to provide better control and prevention of virus shedding after NDV infection. The vaccination of broiler chickens with recombinant genotype VII live vaccine reduced oropharyngeal shedding of virus compared to the LaSota vaccine at 3 dpc with a subsequent significant reduction of cloacal shedding compared to LaSota live vaccine at 7 dpc.

CONCLUSION

In conclusion, the results of vaccination efficacy indicated that the genotype-matched vaccine (live genotype VII) to the challenge virus was able to reduce virus shedding significantly as well as provided significant protection against mortality compared to classic antigenically-divergent vaccines (live genotype II, LaSota) in commercial Cobb 500 broiler chickens. However, both vaccines did not confer adequate protection. Therefore, further studies are needed to evaluate more intensive live vaccination regimens or even introduce inactivated NDV vaccines in broiler vaccination programs in order to achieve better protection against currently epidemic vNDV infection.

DECLARATIONS

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Competing interests

All authors have no conflict of interest.

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Author's contribution

All authors equally participated in design, experimental procedure, writing, revised, and reviewing the manuscript.

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Model Estimation for LongitudinalBone Growth Based on Age in Male and Female Commercial Broiler Chickens

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ABSTRACT

Longitudinal bone growth is essential to support rapid body growth in commercial broiler chickens. The present study aimed to determine which simple mathematic model is best suitable for explaining the absolute and the relative bone growth in length (expressed as a ratio of bone length to body weight) as a function of age in male and female commercial broiler chickens over the first 35 days of age. A total of 1,800 broiler chickens (900 males and 900 females) of Cobb 500, Ross 308, and Arbor Acres raised in standard commercial broiler houses were randomly selected for this study. Body weight and the lengths of backbones, third toe, shank bone, and keel bone were individually measured in all chickens at 1, 7, 14, 21, 28, and 35 days of age. Regression analysis (with 4 simple mathematical models including linear, logarithmic, inverse, and exponential) was used to find a suitable model for estimating the absolute and the relative bone growth in length. In addition, an adjusted R^2 was used to assess the model fitting. The results indicated that the absolute bone growth in length linearly increased with age. The adjusted R^2 values for the linear model were 0.973, 0.937, 0.950, and 0.974 for the lengths of the backbones, third toe, shank bone, and keel bone, respectively. However, the relative bone growth in length logarithmically decreased with age. The adjusted R^2 values of the logarithmic model were 0.971, 0.952, 0.957, and 0.905 for the relative length of the backbones, third toe, shank bone, and keel bone, respectively.The present investigation suggests thata linear model is a suitable model for estimating the absolute bone growth in length, but a logarithmic model is a proper model for estimating the relative bone growth in length of commercial broiler chickens.

Key words: Age, Bone growth, Broiler chickens, Mathematical model, Regression

INTRODUCTION

The meat of broiler chickens is a popular and cheap protein source for humans worldwide. To serve a huge demand with affordable cost for consumers, fast-growing strains of commercial broiler chickens are necessary. At present, commercial broiler chickens can reach a market size of 2.2 kg within 35 days of age (Tallentire et al., 2018). The percentages of breast plus leg muscles of these chickens accounts for more than 40% of whole eviscerated carcasses (Kokoszyński and Bernacki, 2008; Sarsenbek et al., 2013). However, due to a very rapid growth rate, fast-growing commercial broiler chickens face several problems or disorders, especially in bone and leg (Cook, 2000; Knowles et al., 2008; Granquist et al., 2019). The skeletonis an important structure that supports the whole body; therefore, bone growth is a key physiological process to ensure a proper supporting system for the whole body of animals. Bone growth has many aspects, such as circumferential growth, weight growth, volume growth, bone mineralization, chemical composition, and structural organization (Rose et al., 1996; Kerschnitzki et al., 2016; Pratt and Cooper, 2018; Sanchez-Rodriguez et al., 2019). Bone growth in length (or longitudinal bone growth) is an important factor that supports the rapid expansion of the body of broiler chickens. Several studies have indicated that absolute rate of longitudinal bone growth is positively associated with the age or weight of broiler chickens (Applegate and Lilburn, 2002; Biesiada-Drzaga et al., 2012; Han et al., 2015; Mabelebele et al., 2017). However, one study indicated that relative bone growth in length, expressed as a ratio of bone length to body weight, was negatively associated with body weight gain in broiler chickens (Shim et al., 2012). This ratio is a useful variable to assess the rate of bone length compared to the rate of body weight gain as a function of age. To better understand the bone growth in length as a function of age

in broiler chickens, the objective of this study was to determine which simple mathematical model is suitable for explaining the absolute and the relative bone growth in length in male and female commercial broiler chickens over the first 35 days of the rearing period.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Institutional Animal Care and Use Committee of Khon Kaen University, Khon Kaen, Thailand (ACUC KKU license No. 27/2559).

Animals and housing

The present study was conducted in six broiler houses (three each) from two commercial broiler farms located in Buriram Province, Northeastern Thailand on June 2018 (Latitude: 14° 36' 21.31" N, Longitude: 103° 07' 14.92" E). The year-round outside temperature was approximately 27 °C, and the relative humidity was approximately 75%. Both farms used a tunnel ventilated house with dimensions of 14 × 2.8 × 120 m (width x height x length), resulting in a 1,680 m^2 rearing area. Each house was equipped with a cooling pad on both lateral sides near the front end and 10 large exhaust fans at the rear end. Each house was equipped with four rows of automatic feeding pans, totaling 644 pans, and five rows of the drinkers, totaling 2,366 heads. The stock density was 11-12.5 heads per square meter, resulting in 18,000 to 21,000 birds per house. Each house was considered for both sexes but with a single breed. Chickens were reared in floor pens. The floor was made from concrete cement and covered with a 5 cm thick layer of new rice husk for each growing cycle. Diet, feeding, vaccination, husbandry, and care of the broiler chickens were under standard conditions depending on the age of chickens recommended by the broiler breeder companies. Briefly, feed and water were provided *ad libitum* throughout the rearing cycle. Starter, grower, and finisher feed were used for chickens at aged 1-21 days, 22-32 days, and 33 days until the end of the rearing period, respectively. All chickens were vaccinated against Newcastle disease and infectious bronchitis according to a routine vaccination program. In addition, the chickens were regularly inspected for health status by a veterinarian. The flocks and the farms had no history of disease outbreaks. The temperature in the poultry house was controlled by using a heater or tunnel ventilation system depending on the age of chickens to provide the optimal conditions for birds according to the guidelines for commercial broilers.

Study design, sampling, and outcome measurements

This observational study was designed based on three chick characteristics as follows: age with six levels (1, 7, 14, 21, 28, and 35 days of age), sex with two categories(male and female), and breed with three types (Cobb 500, Ross 308, and Arbor Acres). Therefore, 36 subgroups were included and 50 healthy broiler chickens were randomly selected from the flocks for each subgroup. This resulted in a total sample of 1,800 broiler chickens. All samples were randomly selected from the flocks around the middle of the commercial broiler house (approximately 60 meters from the front end of the house). All measurements were made non-invasively on living animals. For a selected chicken, the body weight was measured with a digital weight scale. Moreover, the length of the backbones, a third toe, a shank bone (a tarsometatarsus), and a keel bone was measured with a Vernier caliper. All variables were measured in living birds using two people (one held the bird gently and another one made the measurement). After birds were measured, they were returned to their flocks. The total length of the backbones (including thoracic, lumbosacral, and caudal vertebrae) was measured from the junction of the last cervical vertebra to the distal end of the last caudal vertebra. The third toe was measured from the proximal end (metatarso-digital joint) to the tip of the claw. The shank bone was measured from the proximal end to the distal end. The keel bone was measured from the proximal end to the distal end. The original measurement scale was in grams for body weight and in millimeters for the bones length.

Statistical analysis

All data from each variable were verified and checked for normality. The mean and standard deviation from each variable for males and females at six different ages (1, 7, 14, 21, 28, and 35 days) of broiler chickens were calculated. A mean difference of each variable was compared using an independent sample t-test. The relative bone growth in length was calculated as a ratio of bone length to body weight. To avoid many decimals and for ease of interpretation, this ratio was expressed in cm/kg. Regression analysis with four mathematical models was used to estimate the absolute and relative bone growth in length as a function of age in broiler chickens. These mathematical models included linear, logarithmic, inverse, and exponential models. The mathematical equation for each model was as follows.

Linear model: $Y = b0 + (b1 * t)$.

*Logarithmic model: Y = b0 + (b1 * log (t)).*

Inverse model: *Y = b0 + (b1 / t).*

Exponential model: *Y = b0 * (e** (b1 * t)).*

(*Y* stands for the dependent variable; *b0* for the intercept; *b1* for the slope; *e* for an irrational constant (approximately equal to 2.718); and *t* for the independent variable (age of chickens)). A p-value of <0.05 was considered statistically significant. SPSS version 17 (SPSS Inc, Chicago, IL) was used for all statistical analysis.

RESULTS

The mean and standard deviation of the body weight of male and female broiler chickens at 1, 7, 14, 21, 28, and 35 days of age is shown in table 1. The difference in mean body weight was significantly higher in males than in females starting from days 7 through 35. The effect sizes of the mean differences were 4.9 g at 7 days to 260 g at 35 days of age.

Comparison of the absolute and the relative bone growth in length between the studied male and female broiler chickens for the backbones, the third toe, the shank bone, and the keel bone are presented in tables 2-5. At 28 and 35 days of age, the absolute length of the backbones was significantly greater in males than in females; however, the relative length was significantly smaller in males compared to females at 7, 14, 21, 28, and 35 days of age (Table 2). The absolute length of the third toe was significantly greater in males than in females at 21, 28, and 35 days of age; however, the relative length was significantly smaller in males than in females at 7, 14, 21, 28, and 35 days of age (Table 3). The absolute length of the shank bone was significantly greater in males than in females at 14, 28, and 35 days of age; however, the relative length was significantly smaller in males than in females at 7, 21, 28, and 35 days of age (Table 4). The absolute length of the keel bone was significantly greater in males than in females at 14, 21, 28, and 35 days of age; however, the relative length was significantly smaller in males than in females at 7, 14, 21, 28, and 35 days of age (Table 5).

Regression analysis of linear, logarithmic, inverse, and exponential models for estimating the association between the absolute bone growth in length and the age of the broiler chickens are shown in table 6. The regression coefficient of the absolute bone length as a function of age was significant for all models and for all the studied bones ($p<0.001$) (Table 6). However, the adjusted R^2 was the greatest for the linear model for all bone types (0.973, 0.937, 0.950, and 0.974 for backbones, third toe, the shank bone, and the keel bone; respectively) (Table 6). Visually, a linear model was a better model than a logarithmic model in fitting the data for the absolute bone growth in length in the broiler chickens (Figure 1A-4A). In the linear model, a regression coefficient for age was positively maximal for the keel bone (3.46) and positively minimal for the third toe (1.13) (Table 6).

Regression analysis with four different models (linear, logarithmic, inverse, and exponential models) for estimating the association between the relative bone growth in length and age of broiler chickens are presented in table 7. The regression coefficient of the relative bone length as a function of age was significant for all the models and for all the bones ($p<0.001$) (Table 7). However, the adjusted R^2 was the greatest for the logarithmic model for most bone types (0.971 for backbones, 0.952 for the third toe, 0.957 for the shank bone, and 0.905 for the keel bone) (Table 7). Visually, the logarithmic model was a better model than the linear model in fitting the data for the relative bone growth in length in the broiler chickens (Figure 1B-4B). In the logarithmic model, a regression coefficient for age was negatively maximal for the keel bone (-31.28) and negatively minimal for the third toe (-13.44) (Table 7).

Table 1. Body weight of broiler chickens at different ages

Body weight (g) / Age of chickens (day)	Male Mean ± SD	Female Mean ± SD	MD (95% CI)	p-value
1	43.6 ± 2.5	44.0 ± 2.7	-0.4 (-1.0, 0.2)	0.181
7	180.9 ± 11.6	176.0 ± 15.4	4.9 (1.8, 8.0)	0.002
14	479.5 ± 15.7	462.0 ± 12.8	17.5 (14.3, 20.8)	<0.001
21	959.6 ± 15.1	902.2 ± 16.1	57.3 (53.8, 60.9)	<0.001
28	1575.8 ± 28.6	1435.3 ± 30.7	140.5 (133.7, 147.2)	<0.001
35	2282.6 ± 43.8	2022.8 ± 55.2	259.8 (248.5, 271.2)	<0.001

CI: confidence interval, MD: mean difference, SD: standard deviation

Table 2. The absolute length and relative length (expressed as a ratio of bone length to body weight) of backbones in male and female broiler chickens at different ages

Age (days)	Absolute length of backbones (mm)				Relative length of backbones (cm/kg)			
	Male Mean ± SD	Female Mean ± SD	MD (95% CI)	p-value	Male Mean ± SD	Female Mean ± SD	MD (95% CI)	p-value
1	50.8 ± 2.4	50.0 ± 2.0	0.8 (0.3, 1.3)	0.001	116.9 ± 7.8	114.0 ± 7.9	2.9 (1.1, 4.6)	0.002
7	79.6 ± 4.8	80.6 ± 3.6	-1.0 (-2.0, 0.0)	0.042	44.2 ± 3.6	46.2 ± 4.7	-2.0 (-2.9, -1.1)	<0.001
14	100.0 ± 9.1	103.9 ± 5.4	-3.8 (-5.5, -2.1)	<0.001	20.9 ± 1.9	22.5 ± 1.3	-1.6 (-2.0, -1.3)	<0.001
21	118.6 ± 3.8	119.9 ± 5.7	-1.3 (-2.4, -0.2)	0.021	12.4 ± 0.4	13.3 ± 0.6	-0.9 (-1.0, -0.8)	<0.001
28	146.4 ± 5.8	143.2 ± 4.2	3.2 (2.0, 4.3)	<0.001	9.3 ± 0.4	10.0 ± 0.4	-0.7 (-0.8, -0.6)	<0.001
35	169.6 ± 7.5	165.2 ± 5.8	4.4 (2.9, 6.0)	<0.001	7.4 ± 0.4	8.2 ± 0.3	-0.7 (-0.8, -0.7)	<0.001

CI: confidence interval, MD: mean difference, SD: standard deviation

Table 3. The absolute length and the relative length (expressed as a ratio of bone length to body weight) of the third toe in male and female broiler chickens at different ages

Age (days)	Absolute length of the third toe (mm)				Relative length of the third toe (cm/kg)			
	Male Mean ± SD	Female Mean ± SD	MD (95% CI)	p-value	Male Mean ± SD	Female Mean ± SD	MD (95% CI)	p-value
1	21.4 ± 2.2	21.5 ± 1.7	-0.1 (-0.5, 0.4)	0.814	49.3 ± 6.5	49.0 ± 5.8	0.3 (-1.1, 1.7)	0.667
7	32.4 ± 1.5	32.6 ± 1.4	-0.1 (-0.5, 0.2)	0.444	18.0 ± 1.7	18.7 ± 2.3	-0.7 (-1.1, -0.2)	0.005
14	41.5 ± 3.1	41.1 ± 2.2	0.4 (-0.3, 1.0)	0.248	8.7 ± 0.7	8.9 ± 0.5	-0.3 (-0.4, -0.1)	0.001
21	46.2 ± 1.6	45.8 ± 1.7	0.4 (0.0, 0.8)	0.033	4.8 ± 0.2	5.1 ± 0.2	-0.3 (-0.3, -0.2)	<0.001
28	56.3 ± 3.3	54.3 ± 3.2	2.0 (1.2, 2.7)	<0.001	3.6 ± 0.2	3.8 ± 0.3	-0.2 (-0.3, -0.2)	<0.001
35	62.7 ± 5.0	59.4 ± 4.1	3.3 (2.3, 4.3)	<0.001	2.7 ± 0.2	2.9 ± 0.2	-0.2 (-0.2, -0.1)	<0.001

CI: confidence interval, MD: mean difference, SD: standard deviation

Table 4. The absolute length and relative length (expressed as a ratio of bone length to body weight) of the shank bone in male and female broiler chickens at different ages

Age (days)	Absolute length of the shank bone (mm)				Relative length of the shank bone (cm/kg)			
	Male Mean ± SD	Female Mean ± SD	MD (95% CI)	P-value	Male Mean ± SD	Female Mean ± SD	MD (95% CI)	P-value
1	29.1 ± 2.0	29.0 ± 1.4	0.1 (-0.3, 0.5)	0.576	66.8 ± 5.3	66.0 ± 4.3	0.9 (-0.2, 1.9)	0.127
7	39.1 ± 1.7	39.1 ± 1.4	0.0 (-0.3, 0.4)	0.970	21.7 ± 1.8	22.4 ± 2.4	-0.7 (-1.2, -0.2)	0.005
14	54.2 ± 3.5	52.5 ± 2.4	1.7 (1.0, 2.4)	<0.001	11.3 ± 0.8	11.4 ± 0.6	-0.1 (-0.2, 0.1)	0.478
21	63.1 ± 8.2	61.9 ± 8.1	1.2 (-0.7, 3.0)	0.214	6.6 ± 0.8	6.9 ± 0.9	-0.3 (-0.5, -0.1)	0.003
28	79.0 ± 3.8	76.3 ± 3.5	2.8 (2.0, 3.6)	<0.001	5.0 ± 0.2	5.3 ± 0.2	-0.3 (-0.3, -0.2)	<0.001
35	89.8 ± 4.0	84.3 ± 3.8	5.5 (4.7, 6.4)	<0.001	3.9 ± 0.2	4.2 ± 0.2	-0.2 (-0.3, -0.2)	<0.001

CI: confidence interval, MD: mean difference, SD: standard deviation

Table 5. The absolute length and the relative length (expressed as a ratio of bone length to body weight) of keel bone in male and female broiler chickens at different ages

Age (days)	Absolute length of the keel bone (mm)				Relative length of the keel bone (cm/kg)			
	Male Mean ± SD	Female Mean ± SD	MD (95% CI)	P-value	Male Mean ± SD	Female Mean ± SD	MD (95% CI)	p-value
1	23.3 ± 4.4	23.6 ± 4.6	-0.4 (-1.4 0.7)	0.500	53.7 ± 11.7	54.2 ± 12.8	-0.5 (-3.3, 2.3)	0.729
7	52.4 ± 2.7	52.4 ± 3.4	0.0 (-0.7, 0.7)	0.911	29.1 ± 2.8	30.1 ± 4.0	-1.0 (-1.8, -0.2)	0.013
14	79.7 ± 3.7	78.2 ± 4.0	1.5 (0.6, 2.4)	0.001	16.6 ± 0.0	16.9 ± 1.0	-0.3 (-0.5, -0.1)	0.007
21	105.1 ± 3.7	102.9 ± 3.7	2.1 (1.3, 3.0)	<0.001	11.0 ± 0.4	11.4 ± 0.5	-0.5 (-0.6, -0.4)	<0.001
28	127.9 ± 5.2	123.4 ± 4.9	4.5 (3.3, 5.6)	<0.001	8.1 ± 0.3	8.6 ± 0.3	-0.5 (-0.6, -0.4)	<0.001
35	143.9 ± 5.2	138.2 ± 6.2	5.7 (4.4, 7.0)	<0.001	6.3 ± 0.2	6.8 ± 0.3	-0.5 (-0.6, -0.5)	<0.001

CI: confidence interval, MD: mean difference, SD: standard deviation

Table 6. Regression analysis for estimating the relationship between the absolute bone growth in length and age of broiler chickens

Variables	Model equation	Adjusted R^2	Constant		Age (day)		p-value for age
			B	SE	B	SE	
Length of backbones (mm)	Linear	0.973	52.14	0.28	3.31	0.01	<0.001
	Logarithmic	0.838	38.04	0.84	30.01	0.31	<0.001
	Inverse	0.561	129.25	0.73	-84.13	1.75	<0.001
	Exponential	0.924	57.51	0.27	0.03	0.00	<0.001
Length of third toe (mm)	Linear	0.937	23.04	0.15	1.13	0.01	<0.001
	Logarithmic	0.848	17.63	0.28	10.45	0.10	<0.001
	Inverse	0.589	49.51	0.24	-29.84	0.59	<0.001
	Exponential	0.890	24.38	0.12	0.03	0.00	<0.001
Length of shank bone (mm)	Linear	0.950	27.58	0.20	1.73	0.01	<0.001
	Logarithmic	0.776	21.21	0.52	15.25	0.19	<0.001
	Inverse	0.485	67.22	0.42	-41.27	1.00	<0.001
	Exponential	0.933	30.77	0.13	0.03	0.00	<0.001
Length of keel bone (mm)	Linear	0.974	26.40	0.28	3.46	0.01	<0.001
	Logarithmic	0.868	10.37	0.79	31.92	0.29	<0.001
	Inverse	0.585	107.42	0.74	-89.76	1.78	<0.001
	Exponential	0.857	31.21	0.32	0.05	0.00	<0.001

B: unstandardized regression coefficient, SE: standard error

Table 7. Regression analysis for estimating the relationship between the relative bone growth in length (expressed as a ratio of bone length to body weight) and age of broiler chickens

Variables	Model equation	Adjusted R^2	Constant		Age (day)		p-value for age
			B	SE	B	SE	
Backbones to body weight (cm/kg)	Linear	0.679	82.79	0.92	-2.68	0.04	<0.001
	Logarithmic	0.971	111.09	0.35	-31.28	0.13	<0.001
	Inverse	0.939	12.13	0.26	105.37	0.63	<0.001
	Exponential	0.918	85.16	0.98	-0.08	0.00	<0.001
Third toe to body weight (cm/kg)	Linear	0.661	34.89	0.41	-1.15	0.02	<0.001
	Logarithmic	0.952	47.14	0.19	-13.44	0.07	<0.001
	Inverse	0.929	4.59	0.12	45.45	0.30	<0.001
	Exponential	0.923	36.10	0.42	-0.08	0.00	<0.001
Shank bone to body weight (cm/kg)	Linear	0.639	45.85	0.56	-1.50	0.03	<0.001
	Logarithmic	0.957	62.73	0.24	-17.95	0.09	<0.001
	Inverse	0.962	5.67	0.12	61.63	0.29	<0.001
	Exponential	0.902	45.56	0.59	-0.08	0.00	<0.001
Keel bone to body weight (cm/kg)	Linear	0.726	43.35	0.39	-1.26	0.02	<0.001
	Logarithmic	0.905	54.32	0.28	-13.74	0.11	<0.001
	Inverse	0.792	11.34	0.22	44.02	0.53	<0.001
	Exponential	0.942	46.21	0.35	-0.06	0.00	<0.001

B: unstandardized regression coefficient, SE: standard error

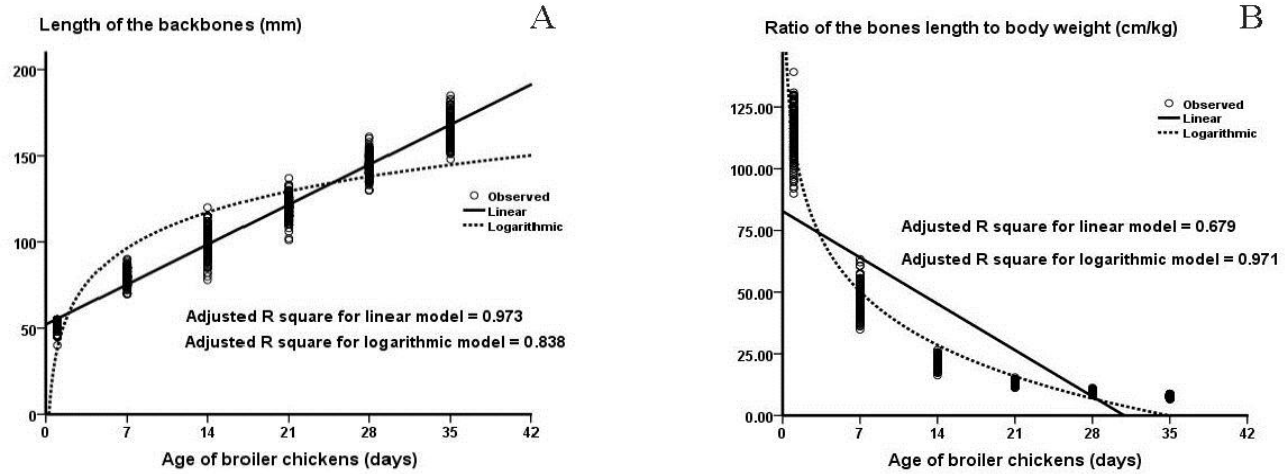

Figure 1. Regression analysis using linear and logarithmic models for the absolute (**A**) and the relative (**B**) bone growth in length (expressed as a ratio of bone length to body weight) of the backbones in the broiler chickens during 35 days rearing period.

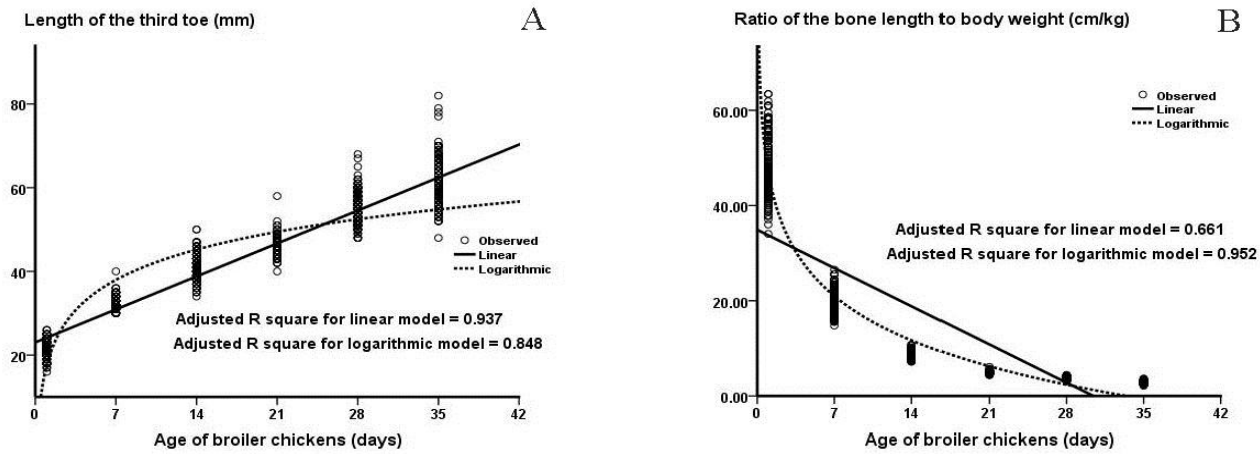

Figure 2. Regression analysis using linear and logarithmic models for the absolute (**A**) and the relative (**B**) bone growth in length (expressed as a ratio of bone length to body weight) of the third toe in the broiler chickens during 35 days rearing period.

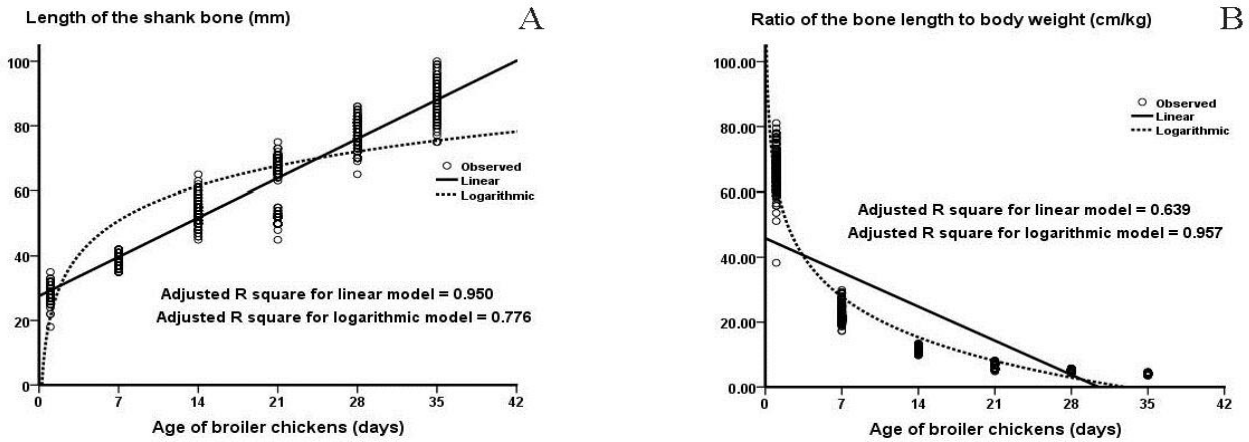

Figure 3. Regression analysis using linear and logarithmic models for the absolute (**A**) and the relative (**B**) bone growth in length (expressed as a ratio of bone length to body weight) of the shank bone in the broiler chickens during 35 days rearing period.

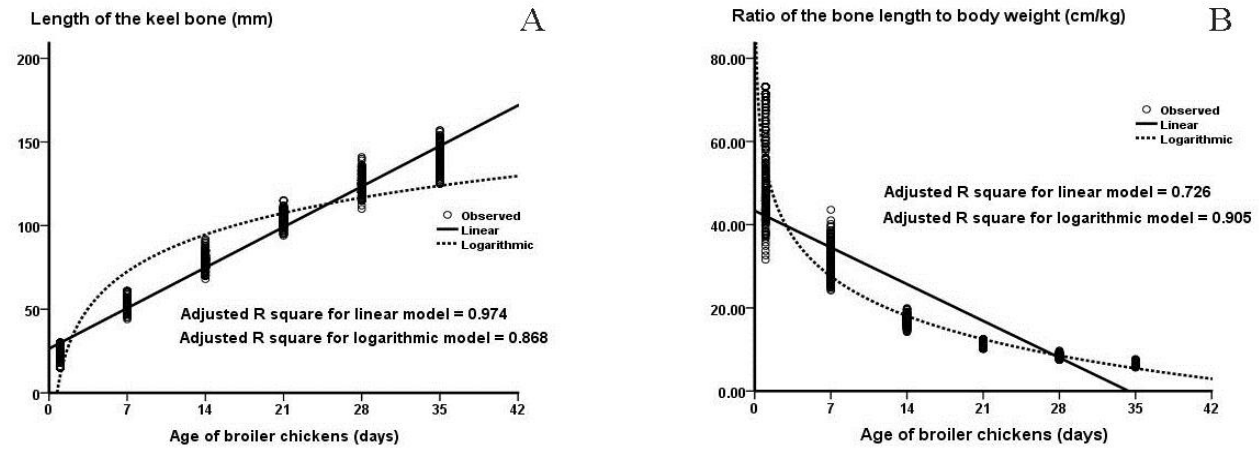

Figure 4. Regression analysis using linear and logarithmic models for the absolute (**A**) and the relative (**B**) bone growth in length (expressed as a ratio of bone length to body weight) of the keel bone in the broiler chickens during 35 days rearing period.

DISCUSSION

This study indicated that a different mathematical model was acceptable for explaining the absolute and relative bone growth in length as a function of age in commercial broiler chickens. A linear model is more suitable for explaining the absolute bone growth in length in commercial broilers because an adjusted R^2 value (indicating model fitting) of the linear model was higher than that of the other models. The adjusted R^2 values for the linear model of the absolute bone growth in length ranged from 0.937 for the third toe and to 0.974 for the keel bone, which were higher than those of the other models for all of the studied bones (Table 6). This result means that the age of the chickens can explain approximately 93.7% and 97.4% of the variability in bone growth in length for the third toe and the keel bone, respectively. However, it appeared that a logarithmic model is more suitable for explaining the relative bone growth in length in commercial broiler chickens. Adjusted R^2 values for assessing a logarithmic model fitting of the relative bone growth in length ranged from 0.905 for the keel bone to 0.971 for the backbones, which was higher than those of the other models for most of the studied bones (Table 7). This finding indicates that the age of the chickens can explain approximately 90.5% and 97.1% of the variability in bone growth in length for the keel bone and the backbones, respectively.

In the linear model, the absolute bone growth in length linearly increased with age for all of the studied bones. The rate for the absolute bone growth in length was the highest for the keel bone (the regression coefficient = 3.46) and was the lowest for the third toe (the regression coefficient = 1.13) (Table 6). This outcome meant that the keel bone growth in length increased from 29.9 mm at 1 day to 147.5 mm at 35 days of age, but the third toe growth in length increased from 24.2 mm at 1 day to 62.6 mm at 35 days of age. Several studies have evaluated longitudinal bone growth in broiler chickens (Applegate and Lilburn, 2002; Biesiada-Drzaga et al., 2012; Shim et al., 2012; Han et al., 2015; Mabelebele et al., 2017). The results of previous studies were similar to the obtained results in the current study. That is, the absolute bone growth in length in the broiler chickens was positively increased with age (Biesiada-Drzaga et al., 2012; Han et al., 2015) or with body weight (Applegate and Lilburn, 2002; Paxton et al., 2014; Mabelebele et al., 2017). The present study also indicated that the rate of longitudinal bone growth differed depending on the studied bones. This difference may result in bone length reaching a plateau or maturity at different ages. The statement was also supported by evidence from a previous study, conducted in broiler chickens from hatch to 43 days of age, which reported the length of the femur reached a plateau at 35 days of age, but the length of the tibia reached a plateau later than 43 days of age (Applegate and Lilburn, 2002). In addition, this finding was similar to longitudinal bone growth in other poultry species, such as ducks (Van Wyhe et al., 2012).

In a logarithmic model, the relative bone growth in length logarithmically decreased with age. It was indicated that the relative bone growth in length decreased sharply from 1 day to 14 days of age but slightly decreased from 21 days through 35 days (Figure 1B-4B). The rate of the relative bone length was negative, with the lowest rate for the backbones (regression coefficient = -31.28) and the highest rate for the third toe (regression coefficient = -13.44). Moreover, it was determined that the relative length of the backbones decreased from 109.60 cm/kg (or 1.10 mm/g) at 1 day to 69.23 cm/kg (or 0.69 mm/g) at 28 days of age, but the third toe growth in length decreased from 46.02 cm/kg (or 0.46 mm/g) at 1 day to 15.56 cm/kg (or 0.16 mm/g) at 28 days of age. The obtained results in current work were similar to those of a previous study by Shim et al. (2012), who found the relative bone growth in length decreased with body weight in both fast-growing and slow-growing broiler chickens.

The results obtained in the present article varied in overall comparisons of bone growth in length between male and female broiler chickens for all ages. However, at 28 days and 35 days of age, the absolute bone growth in length was significantly higher in males than in females for all of the studied bones. On the other hand, the relative bone growth in length was significantly lower in males than in females from 7 days through 35 days of age (Tables 2-5). The different results between the absolute and relative bone growth in length implied the importance of different rates of body weight gain between males and females (Table 1). The results from previous studies were also controversial. In one study, there was no significant difference in absolute bone length between male and female broiler chickens (Han et al., 2015). In other studies, male broiler chickens had longer tibias and femurs than female broiler chickens (Bond et al., 1991; Applegate and Lilburn, 2002).

The present study has some limitations. There are several factors that regulate bone growth and strength in poultry (Rath et al., 2000). However, the current investigation focused only on bone growth in length and ignored circumferential growth, weight, and volume of

bones as well as the degree of mineralization which were studied in previous literature (Kerschnitzki et al., 2016; Pratt and Cooper, 2018; Sanchez-Rodriguez et al., 2019). These factors affect the bone strength for supporting the whole body weight. In addition, the age of broiler breeder flocks was not available in the current study. Different ages of broiler breeder may result in differences in bone length and body weight of broiler chickens. Therefore, the aforementioned limitations should be taken into account in the interpretation of bone growth in this study, which should be made carefully.

CONCLUSION

The present study demonstrated that a simple linear model is a suitable model to explain the increase in the absolute bone growth in length as a function of age; however, a logarithmic model is an acceptable model to explain the decrease in the relative longitudinal bone growth as a function of age in commercial broiler chickens.

DECLARATIONS

Acknowledgments

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Consent to publish

Not applicable

Competing interests

The authors have declared that no competing interest exists.

Author's contribution

Saijai, Damnern, and Peerapol participated in study design, data collection, data analysis, writing, and approving the final manuscript.

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Uses of Single Dose Dependent and Relative Potency Assays for Evaluation of Inactivated Fowl Cholera Vaccine

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ABSTRACT

Fowl cholera is a septicemic respiratory complex caused by *Pasteurella multocida*, widely distributed in poultry and other avian species and of major economic importance. A total of 37 different inactivated *Pasteurella multocida* vaccines from different sources either locally prepared or imported from different sources were comparatively tested for relative potency following both single dose and booster dose vaccination assays. The study objective was to minimize the time factor exhausted in the evaluation processes of the inactivated fowl cholera vaccines. So it is planned to compare between single and booster dose vaccinations and their related potency. Correlation between protection associated with the single dose and booster dose vaccination were evaluated and average requirement for protection was 43.7% in single dose vaccination assay compared to 76.2 % associated with booster dose vaccination assay. In the same concern, the correlation between both assays for the seroconversion was estimated using ELISA and the minimum requirement was 1.8× cut off value in the single dose vaccination assay compared to 2.25× cut off value in the booster dose vaccination assay. In conclusion, single dose vaccination assay could be valuable in the evaluation of inactivated fowl cholera vaccines through determination of protection indices and/or estimation of humoral immune response if the above mentioned data is considered.

Key words: Challenge and chicken, ELISA, Inactivated vaccine, *Pasteurella multocida*, Single dose

INTRODUCTION

Respiratory diseases are one of the major causes of economic losses to poultry industry. Fowl cholera is a septicemic respiratory complex caused by *Pasteurella multocida*, where it is highly common and widely distributed disease of poultry and other avian species (Rhoades et al., 1989; Xiao et al., 2015). *P. multocida* infections result in considerable losses to layer and breeder flocks in poultry industries worldwide. *P. multocida* lipopolysaccharide (LPS) is a primary stimulator of the host immune response and a critical determinant of bacterin protective efficacy (Harper et al., 2016). The disease can express itself in an acute or a chronic form. In the acute form, the clinical signs are seen only in the few hours before death as fever, ruffled feathers, mucus discharge from mouth, diarrhea and increased breathing rate. The chronic form of the disease can follow an acute stage or may be the only form of the disease present in the flock. Signs of this form generally linked to localized infection at wattles, sinuses, leg or wing joints, swollen eyes, twisted neck, rales and pin headed necrotic foci in the liver with a septicemic picture (Glisson et al., 2008; Akhtar et al., 2016).

Fowl cholera can be prevented by eliminating all reservoirs of infections and then preventing the re-entry of the organism into the property. Implementation of standard good management practices, effective sanitation regime and good biosecurity program will help prevention of fowl cholera (Blackall, 2003). Vaccination is considered as one of the common preventive measures worldwide to reduce the prevalence and incidence of disease (Kardos and Kiss, 2005). *Pasteurella multocida* exists in 16 different serovars and the most common serovars associated with fowl cholera outbreaks are serovars 1, 3 and 4. *P. multocida* vaccines based mainly on inactivated cells of *P. multocida* (Glisson et al., 2008). Evaluation and quality control of the efficacy of this vaccine are based mainly on vaccination challenge test by which the protective indices are estimated (OIE, 2017).

The immune system defends the organisms against infectious diseases and one of the major immunological

defense mechanism is the humoral immune response, which is mediated by serum antibodies secreted by B cell (Weigend et al., 1997). Serological testing is a useful tool in explanation of immune status of the birds and the Enzyme Linked Immunosorbent Assay (ELISA) have been used to quantify antibodies against p. multocida, employing an array of antigens such as Outer Membrane Protein (Dogra et al., 2015)

The objective of the present work was to study the availability of using single dose vaccination assay in through determination of protection percent and humoral immune response in comparison to the booster dose vaccination assay of *P. multocida* inactivated vaccine in chickens.

MATERIALS AND METHODS

Pasteurella multocida vaccines

A total of 37 different inactivated *P. multocida* vaccinebatches yearling 2012 up to 2016, from different manufacturers sources either locally prepared or imported from abroad were tested by vaccination challenge assay method using virulent *P. multocida*in parallel to serological evaluation using ELISA.

Pasteurella multocida strains

Virulent *Pasteurellamultocida*serovars 1, 3 and 4 were used to perform challenge test. These serovarswere supplied from the reference strain bank, CLEVB (Central Laboratory for the Evaluation of Veterinary Biologics).

Laboratory animals

Chickens

A total of 120 Hy-line Specific Pathogen Free (SPF) chickens aging 6-8 weeks were used for each fowl cholera vaccine batch tested to perform this study which starting from 2012 up to 2016. This birds were divided into three groups, the first one comprised 45 birds and received only one dose then challenged and serologically tested, the second group comprised also 45 birds and received both primary dose and three weeks later received a booster dose then challenged and serologically tested and finally the third group were 30 birds kept as negative unvaccinated group. All birds were vaccinated with the corresponding fowl cholera vaccine batch (0.5 ml/dose/chickens) subcutaneously (OIE 2017).

Swiss mice

Six Swiss mice weighed about 20-25 gram, two for each *P. multocida* serovar were inoculated with the stock culture of *P. multocida*. This was done before every challenge test to rebuild the virulence of *P. multocida* serovars in a dose of 100500 CFU/ mouse intraperitoneally (OIE, 2017).

Blood samples

Twenty blood samples were collected from the jugular vein for each group per each tested batch of vaccines three weeks post vaccination in case of single dose vaccination assay or three weeks post the second dose of vaccination in case of booster dose vaccination assay, then sera were separated to be tested using ELISA.

Challenge test

The vaccinated birds were challenged with 2×10^2 to 3×10^2 CFU/ challenge dose from the different regained virulent *P. multocida* strains (15 vaccinated and five unvaccinated birds/each serovar) three weeks post vaccination in case of single dose vaccination assay or three weeks post the second dose of vaccination in case of booster dose vaccination assay. Mortalities were observed, recorded and re-isolation of the challenge strain were done from the internal organs (liver and heart blood) of dead cases and the Protective Indices (PI) were calculated using the following formula described by Timms and Marshall (1989).

$$PI = \frac{\%\ (M\ \&\ PML)\ controls - \%\ (M\ \&\ PML)\ vaccinated \times 100}{\%\ (M\ \&\ PML)\ controls}$$

Where PI is the protective indices, M is the mortality and PML is the post-mortem lesions.

ELISA

ELISA was conducted on serum samples collected from all groups in different tested batches and the test performed according to standard procedures of the two different commercial kits used. The first one is *P. multocida* antibody test kit (Synbiotics Corporation, Cat. No.96-6527, USA) referred in this study as kit 1 while the second kit is *P. multocida* antibody test kit (IDEXX Laboratories. Inc., Cat. No. 99-09251, Netherland) which referred in this study as kit 2. ELISA was performed and interpreted as directed by the manufacturers.

Statistical analysis

Correlation coefficient which is the reciprocal relation between two or more variables was calculated and analyzed using SPSS program version 21 (2012). A statistic representing how closely two variables co-vary, it can vary from -1 (perfect negative correlation) through 0 (no correlation) to +1 (perfect positive correlation).

Ethical approval

All the procedures have been carried out in accordance with the guidelines laid by institutional Ethics committee of central laboratory for evaluation of veterinary biologics (CLEVB) and in accordance with local lows and regulations.

RESULTS

Generally, fowl cholera vaccines are evaluated by sterility, safety and potency tests. Potency testing depends mainly on challenge test and determination of humoral immune response by ELISA as shown in table 1. A total of 32 out of 37 fowl cholera vaccine batches were tested and indicated satisfactory results for approval to be used in the poultry farms according to the Egyptian standards for evaluation of veterinary biologics (2004). According to the protection level obtained, the tested fowl cholera vaccine batches was grouped into seven categories. The protection percent in the first group comprises 6 batches out of 32 and was 41% in case of single dose vaccination assay compared with 70% in case of booster dose vaccination assay, in the second group comprises 3 batches out of32 was 42% and 72%, the third group comprises 11 batches (the highest average number of tested batches) out of 32 was 43% and 75%, the fourth group comprises 2 batches out of 32 was44% and 76%, the fifth group comprises 7 batches out of 32 was 44% and 78%, the sixth group comprises 2 batches out of 32 was45% and 80% and the last seventh group comprises only one batch out of 32 was 47% and 82% in case of single and booster dose assays for each group respectively. On the other hand, table 1 also showed a comparison between the humeral immune response expressed ELISA mean titer for the same batch group at the same protection level. It was noticed that, the antibody titers at the protection level of 41% was parallel to 272 and 717 ELISA antibody titer for both types of ELISA kits respectively in case of single dose vaccination assay while it was 341 and 896 at the protection level of 70% in case of booster dose vaccination assay. Also it is clear that the antibody titer was increased as the protection level increased in a harmonious manner for both assays at all level of protections.

Table 1. Enzyme linked immuonosorbent assay mean titers and protection percent in Hy-line specific pathogen free chickens aging 6-8 weeks vaccinated with either single or booster dose vaccination assays of the satisfactory tested inactivated fowl cholera vaccines

No. of tested vaccines batches	Single dose vaccination assay						Booster dose vaccination assay					
	ELISA Mean Titer		Protection mean percent against *P. multocida* serovar				ELISA Mean Titer		Protection mean percent against *P. multocida* serovar			
	Kit 1	Kit 2	1	3	4	Mean	Kit 1	Kit 2	1	3	4	Mean
6	272	717	40	43	40	41	341	896	70	71	70	70
3	290	738	41	42	43	42	362	922	70	73	74	72
11	298	766	42	42	45	43	373	958	77	73	75	75
2	303	883	44	44	44	44	379	1104	78	74	74	76
7	325	914	44	43	45	44	406	1143	78	81	79	78
2	330	926	44	46	45	45	412	1157	80	81	80	80
1	348	954	48	47	46	47	435	1192	85	83	82	83
Total 32	309	843		Mean		43.7	387	1053		Mean		76.2

No: number, P: *Pasturella*, Kit 1: *Pasturella Multocida* ELISA KIT (Synbiotics, USA); Kit 2: *Pasturella Multocida* ELISA kit (IDEXX, Netherland)

By the same manner, table 2 illustrated that five fowl cholera vaccine batches out of 37 are evaluated as unsatisfactory, according to ESEVB (2004) where it got a protection level lower than 70% which is the minimum requirement for protection starting with 60% protection in two vaccine batches, 48%, 47% and 45% protection with one vaccine batch for each. Meanwhile, the protection achieved by the same batches when used only in single dose were 32%, 29%, 27% and 25% respectively.

As regards to the ELISA antibody titer of such unsatisfactory resulted batches, the corresponding antibody titers were 292 and 767 ELISA antibody titer with the protection rate 60% compared to 234 and 614 ELISA antibody titer with protection rate 32% in case of booster dose and single dose vaccination assays respectively. Also antibody titer decreased as the protection percent decreased in a parallel manner matched the immune status of the tested vaccine and birds in the rest unsatisfactory results of the tested batches.

Table 3, showed the average responses of birds vaccinated with either single or booster dose vaccination assays regarding both humoral responses and protection

obtained. The average protection percent of all tested satisfactory batches was 76.2 with the booster dose compared to 43.7 with the single dose vaccination assay. Meanwhile, the average of measured ELISA antibody titer was 387 and 1053 compared to 309 and 843 for both kits and both assays respectively. On the other hand, the average protection percent of all tested unsatisfactory batches was 50 with the booster dose compared to 29 with the single dose vaccination assay. At the same time, the average of measured antibody titer was 242 and 619 compared to 194 and 495 for both kits and both assays respectively.

Table 2. Enzyme linked immuonosorbent assay mean titers and protection percent in Hy-line specific pathogen free chickens aging 6-8 weeks vaccinated with either single or booster dose vaccination assays of the unsatisfactory tested inactivated fowl cholera vaccines

No. of tested vaccines batches	Single dose vaccination assay						Booster dose vaccination assay					
	ELISA Mean Titer		Protection mean percent against *P. multocida* serovar				ELISA Mean Titer		Protection mean percent against *P. multocida* serovar			
	Kit 1	Kit 2	1	3	4	Mean	Kit 1	Kit 2	1	3	4	Mean
2	234	614	31	33	32	32	292	767	58	62	60	60
1	193	497	30	28	29	29	241	621	50	44	50	48
1	175	471	27	26	28	27	219	589	45	43	53	47
1	174	399	25	24	26	25	217	499	44	43	48	45
Total 5	194	495		Mean		29	242	619		Mean		50

No: number, P: *Pasturella*; Kit 1: *Pasturella Multocida* ELISA KIT (Synbiotics, USA); Kit 2: *Pasturella Multocida* ELISA kit (IDEXX, Netherland)

Table 3. Comparison and the correlation between mean of protection and ELISA titer afforded by single and booster fowl cholera vaccination in Hy-line specific pathogen free chickens aging 6-8 weeks

Results	No of tested batches	Single dose vaccination Assay			Booster dose vaccination Assay		
		Protection Mean (%)	ELISA Mean Titer		Protection Mean (%)	ELISA Mean Titer	
			Kit 1	Kit 2		Kit 1	Kit 2
Satisfactory	32	43.7	309	843	76.2	387	1053
Unsatisfactory	5	29	194	495	50	242	619

No: number; Kit 1: *Pasturella Multocida* ELISA KIT (Synbiotics, USA); Kit 2: *Pasturella Multocida* ELISA kit (IDEXX, Netherland)

DISCUSSION

Fowl cholera is a highly contagious and economically important disease of poultry worldwide. It is extremely important for poultry producers to be able to get a good vaccine against all poultry pathogens especially that they have great effect on this industry like fowl cholera. Evaluation of the efficacy of inactivated *P. multocida* or fowl cholera vaccine depends mainly on testing of its potency using vaccination- challenge test prior to sale and distribution (OIE, 2017).

Results of this study compared between two different vaccination assays either single dose or booster dose vaccination assays for the evaluation of inactivated fowl cholera vaccine using vaccination–challenge test and monitoring the immune response through determining the antibody titer against the inoculated vaccine using ELISA.

Depending on the minimum requirement of protection (70%) which should be obtained after booster dose of vaccination of fowl cholera vaccine (Egyptian standards for evaluation of veterinary biologics, 2004), the satisfactory protection obtained in this study varies from 70% up to 83% giving rise a protection of 76.2 % as an average in case of booster dose vaccination assay compared to 41% up to 47% giving rise a protection of 43.7 as an average in case of single dose vaccination assay. Meanwhile the average correlated ELISA antibody titer at this protection level was 387 and 1053 ELISA antibody titerin case of booster dose vaccination assay compared to 309 and 843 ELISA antibody titer obtained with single dose vaccination assay using ELISAkit 1 and 2 respectively. Analytical view of these antibody titer revealed that, it is equal to or more than the 2.25× and 1.8× the calculated cut off value of both used kit 1 and kit 2. Also, these titers increased when the protection rate increased and decreased when the protection rate decreased as shown in tables 1 and 2. The same finding was obtained by Akhtar et al. (2016) found that the antibody titer in birds of group A injected with 1ml of vaccine and group B injected with 0.5 ml of vaccine were

4.513 and 4.07 respectively at primary vaccination, and 4.893 and 4.37 respectively after booster vaccination indicating significant increase in the antibody titre as compared to the titre of the primary vaccinaterd birds. The same criteriawere obtained by Jabbri and Moazeni (2005) who stated that fowl cholera vaccine consisted of serotypes 1, 3 and 4 *P. multocida*strains provided 70-100% protection against challenge with homologous strains. Also they found that the trivalent vaccine can induce immunogenic response in vaccinated chickens and so ELISA assay showed a considerable increase in antibody titer after twice vaccination of 6-8 weeks aged chicken. In the same concern, Belloc et al. (2008) assessed vaccine efficacy by measuring serum antibody titers 4 and 8 weeks after a single vaccination with several adjuvants included in *P. multocida* vaccines in chickens. The study concluded that the vaccine formulated with oil adjuvant MantonideISA70, ISA774 and W/O emulsion based on tween/span induced a strong immune response against *P. multocida*. AlsoJabbri and Moazeni (2005) reported that, the antibody measured with ELISA highly correlated with protection against challenge with virulent organisms. In the same concept, Pankaj and Arvind (2013) reported that a blocking ELISA was developed and standardized for the detection of antibodies to *P. multocida* in vaccinated animal. Meanwhile, Gong et al. (2016) detected serum antibody levels using indirect ELISA and reported that serum antibody levels of rPtfA subunit vaccine group and the attenuated live vaccine group were significantly higher than those of PBS group. In addition, there were no difference in antibody levels between the attenuated live vaccine group and rPtfA subunit vaccine group.

Regarding the average protection percent of all tested satisfactory batches, it was 76.2 % with the booster dose compared to 43.7 % with the single dose vaccination assay parallel to the average of measured antibody titer which was 378 and 1053 compared to 309 and 843 ELISA antibody titer for the both used kits and both assays respectively. On the other hand, the average protection percent of all tested unsatisfactory batches was 50 with the booster dose vaccination compared to 29 % with the single dose vaccination assay parallel to the average antibody titer which was 242 and 619 compared to 194 and 495 ELISA antibody titerfor both kits and both assays respectively. Parvin et al. (2011) demonstrated the antibody titers following primary and booster vaccinations for four groups of different breeds of commercial birds and the study concluded that all groups of vaccinated birds induced a significant immune response after primary and booster vaccination without significant difference in antibody titers between different breeds. On the other hand, Perelman et al. (1990) recorded that ELISA test did not appear to be adequate for the evaluation of the degree of protection induced in turkey flocks where turkey poults vaccinated at one day old with inactivated *P. multocida* bacterin showed low titers of antibody as measured by ELISA but turkeys vaccinated at three and six weeks of age responded with a higher antibody titers and were resistant to virulent challenge with *P. multocida*. Akhtar et al. (2016) vaccinated birds with formalin killed fowl cholera bacterin, determined antibody titers using ELISA and challenged birds with virulent strain of *P. multocida* then concluded that the prepared formalin killed fowl cholera vaccine induce protective immune response and conferred protection against challenge protection infection caused by the virulent *P. multocida* strains.

By using a simple calculation regarding the finding of this study (Average protection with booster dose vaccination (76.2 %) and with single dose vaccination assay (43.7 %) and according to the minimum requirement in the ESEVB (2004) for veterinary vaccine evaluation which is 70% with the booster dose vaccination assay, the minimum requirement of protection associated with the single dose vaccination assay is 40.14%.

It may be concluded that, the most important finding from the results of this study is the seroconversion of vaccinated birds with fowl cholera vaccine measured by ELISA concurrently with the protection obtained after challenge with the virulent *P. multocida* strains could be valuable and satisfactory in the evaluation of the efficacy of the fowl cholera vaccines using single dose vaccination assay and/or booster dose vaccination assay. Also, the minimum requirement of protection after challenge with the virulent *P. multocida* strains should be 40.14% or more in case of single dose vaccination assay.

DECLARATIONS

Competing interest

The authors have no conflict of interest.

Consent to publish

The authors declared the consent prior to publication of present article.

Author's contributions

Selim, Fatma, Fatma, Afaf and Abd Elhakim created the idea and the designed the study, collected the data, achieved all statistical analysis and wrote the paper.

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Optimization of Nutrient Medium for *Pediococcus acidilactici* DS15 to Produce GABA

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ABSTRACT

Nutrition is an essential factor for microorganisms to grow and survive. Carbon and nitrogen sources are used in producing primary and secondary metabolites. Gamma-Aminobutyric acid (GABA), a non-coded amino acid, is a secondary metabolite which acts as an inhibitory neurotransmitter of the central nervous system. *Pediococcus acidilactici* DS15 is a bacterium belonging to the order of Lactic Acid Bacteria. This study aimed to determine the effects of nutrients including glutamate, nitrogen and carbon sources on GABA production by *Pediococcus acidilactici* DS15. The tests were carried out using a range of 30 mM, 40 mM, 50 mM, 60 mM, and 70 mM glutamate as inducer and carbon sources in the form of peptone, yeast extract, skim milk, NH_4NO_3, KNO_3, whey tofu and soy milk as nitrogen sources, and then glucose, lactose, maltosa, sucrose, palm, and cane sugar as carbon sources. The best sources of both will be tested with levels 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% for nitrogen and 1, 3, 5, 7, 9, 11, 13 and 15% for carbon. The results of this investigation revealed that the addition of 60 mM glutamate caused the higher amount of GABA production and the best source of nitrogen and carbon for *Pediococcus acidilactici* DS15 were 100% whey tofu and 15% palm sugar, respectively. Production rate of GABA by *Pediococcus acidilactici* DS15 could reach up to 311,485 mg / L.

Key words: Carbon, GABA, Glutamate, Nitrogen, *Pediococcus acidilactici* DS15

INTRODUCTION

Microorganisms are widely used in various industrial sectors. In the livestock fields, microorganisms are associated to health advantages and apply as probiotics and Direct-Fed Microbial (DFM) supplementations (Khan et al., 2016). In addition, they play important roles in feed ingredients processing such as fermentation, production of enzymes or other additive compounds. A short process, high production, and easy to use in the production process are some of the supporting factors for the use of microorganisms (Gurung et al, 2013).

The growth of microorganisms can be successful if nutritional, environmental and other requirements are properly provided. These available nutrients such as carbon and nitrogen in the growth medium will be used to produce primary and secondary metabolites (Thirumurugan et al., 2018). Primary metabolites are formed intracellularly and have an essential function for the survival of microbes, while secondary metabolites can be used as emergency nutrition to survive or to defend themselves in the final phase of growth or death phase (Thirumurugan et al., 2018).

Gamma-Aminobutyric Acid (GABA) is one of the secondary metabolites that can be used as an anti-stress agent for humans or livestock. GABA, a non-proteinogenic amino acid, acts as an inhibitory neurotransmitter of the central nervous system (CNS) (Murray et al, 2003). GABA dilates blood vessels and resulted in lowering blood pressure and are used as a medication for stroke treatment. Moreover, GABA has diuretics, tranquilizer, anti-oxidant, and pain relief effects and regulates the secretion of growth hormone (Hao and Schmit, 1993; Kono and Himeno, 2000; Leventhal et al., 2003).

It is demonstrated that GABA can be also produced by microorganisms such as bacteria, yeast and fungi

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(Dhakal et al., 2012). lactic acid bacteria (LAB) are considered as useful and safe microorganisms that are capable to produce GABA (Li H et al., 2010). *Pediococcus acidilactici* is a LAB which has been used as a starter culture in fermented meat, milk, and vegetable which causes distinctive taste changes, improves cleanliness and extends product shelf life (Mora et al., 1997; Porto et al., 2017). *Pediococcus acidilactici* have also been found in the process of production of traditional food such as dadih (fermented buffalo milk) (Anggraini et al., 2018).

Pediococcus acidilactici DS15 requires nitrogen and carbon to meet their daily needs both for living or producing GABA (Donnell et al., 2001; Savijoki et al., 2006). Most LAB strains usually prefer glucose as a carbon source (Kim et al., 2009). Glucose replacement with fructose, lactose, maltose, arabinose, and galactose can reduce GABA production (Cho et al., 2007). The use of inorganic or organic nitrogen can also affect the growth of LAB. Administration of inorganic nitrogen caused the growth of *Lactobacillus buchneri* WPZ001 to be severely inhibited, but providing organic nitrogen source leads to better growth of *L. buchneri* WPZ001 (Zhao et al., 2015). In addition to nitrogen and carbon nutrients, an inducer of glutamate is also needed to increase GABA production. Addition of exogenous glutamic acid can augment GABA synthesis (Kim et al., 2009). There is less data about nutritional requirements of *Pediococcus acidilactici* DS15 to produce GABA optimally. Therefore, the present study aimed to assess the different media nutrients in term of GABA production by *Pediococcus acidilactici* DS15.

MATERIALS AND METHODS

Isolation of *Pediococcus acidilactici* DS15

Pediococcus acidilactici DS15 was isolated from curds as LAB producing GABA (Anggraini et al., 2018). The bacteria were grown anaerobically at 30^{o}C on MRS broth (Merck, Germany) and stored for further analysis. The experiment was carried out at the Feed Technology Industry Laboratory, Faculty of Animal Science, Andalas University, West Sumatra, Indonesia.

GABA production medium

The nutritional content of media in one liter consists of di-potassium hydrogen phosphate 2 gr, di-ammonium hydrogen citrate 2 gr, sodium acetate 5 gr, magnesium sulphate 0.2 gr, and manganese sulphate 0.02 gr.

Optimization of source and nitrogen levels

As nitrogen sources, GABA-producing LAB used peptone, yeast extract, skim milk, NH4NO3, KNO3, whey tofu, and soy milk. The best results from nitrogen sources are continued with different levels of addition, namely 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%. The results were determined by calculating Optical Density (OD) using a spectrophotometer with a wavelength of 600 nm. Then, GABA production was measured by centrifuging at a speed of 10,000 rpm, temperature 4^{o}C and analyzed using Shimadzu HPLC (Kyoto, Japan) C18 column (250 mm × 4.6 mm I.D., particle size 5 µm / L, Alltech, IL, USA)

Optimization Source and Carbon Levels

The treatment was repeated three times. As carbon sources, *Pediococcus acidilactici* used glucose, lactose, maltose, sucrose, granulated sugar, and palm sugar. The best results from carbon sources are continued with different addition levels, namely 1%, 3%, 5%, 7%, 9%, 11%, 13% and 15%. The results were determined by calculating OD using a spectrophotometer with a wavelength of 600 nm. Then, GABA production was measured by centrifuging at a speed of 10,000 rpm, temperature 4^{o}C and analyzed using Shimadzu HPLC (Kyoto, Japan) C18 column (250 mm × 4.6 mm I.D., particle size 5 µm / L, Alltech, IL, USA).

RESULTS AND DISCUSSION

Optimization of glutamate as an inducer

The effect of giving glutamic acid as an inducer of GABA production and growth of *Pediococcus acidilactici* DS15 is shown in figure 1. Giving glutamic acid with a range of 0,2-1% did not seem to have much influence on growth, where the obtained OD value was 0.443; 0.459; 0.453; 0.452, and 0.445. Whereas for the production of GABA there is a rise along with an increase in the concentration of glutamate acid given. The highest concentration of GABA was observed in the giving of inducer as much as 0,8%, which was 159,047 mg/L, but at the giving of 1%, there was a slight decrease to 158,344 mg/L. Zhong et al. (2019) indicated that L-sodium glutamate at the concentration of 1% had the greatest capacity to enhance the production of GABA compared with the other concentrations by *L. pentosus*, However, the highest mycelium biomass was obtained with L-sodium glutamate at 0.5%.

In the present study, there is an increase in GABA production due to the increase in the amount of glutamate

given that was consistent with the results of a previous study (Komatsuzaki et al., 2005). GABA is synthesized from glutamic acid with the help of Glutamic Acid Decarboxylase (GAD) (Kan et al., 2017) so that with the addition of glutamate, the GAD enzyme will be activated. Bibb (2005) declared that the synthesis of secondary metabolites was triggered by critical conditions of a nutrition or by adding of inducers to a growth medium. GABA synthesis is influenced by the ability of bacteria and also the presence of glutamate in the cell-matrix. The GAD enzyme in LAB is an intracellular enzyme (Huang et al., 2007; Komatsuzaki et al., 2008) that is synthesized as a form of a stress response to an acidic environment (Sanders et al., 1998; Small and Waterman, 1998). L-glutamate concentration can be increased by adding exogenous glutamate acid (Park and Oh, 2005; Seok et al., 2008; Kim et al., 2009; Zhuang et al., 2018), protease to hydrolyze proteins and produce glutamate acid, using LAB to hydrolyze proteins as co-cultures in the fermentation process (Inoue et al., 2003).

Figure 1 shows when the concentration of giving MSG exceed 0,8% resulting in decreased GABA production, therefore, there is an optimal limit of MSG as an inducer, which is 0,8%. This finding is compatible with the study that has been reported excess monosodium glutamate can inhibit GABA production (Tung et al., 2011).

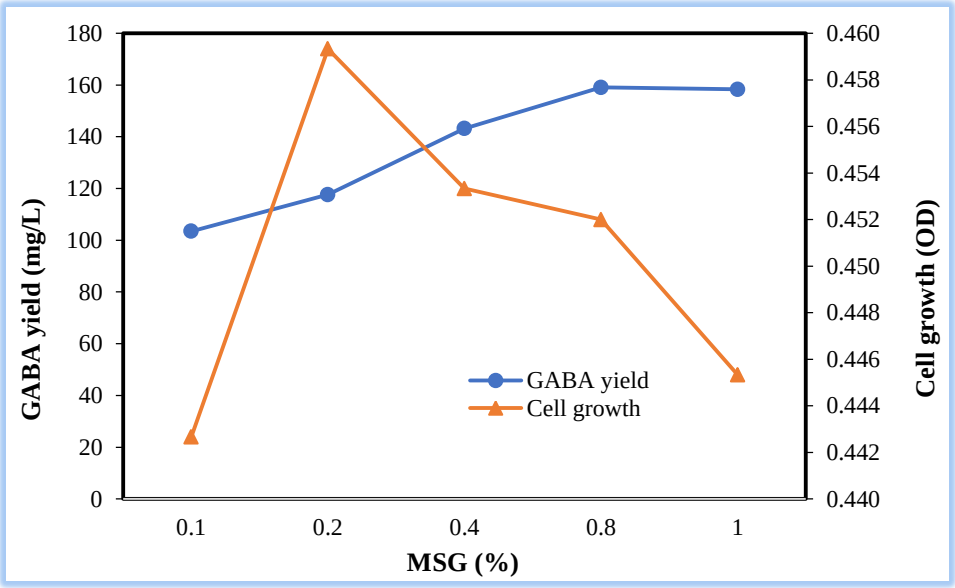

Figure 1. GABA production and growth curve of *Pediococcus acidilactici* DS15 based on glutamate dose

Utilization of nitrogen sources

The uses of various nitrogen sources were investigated to determine optimal GABA production. Figure 2 illustrates that the use of whey as a nitrogen source is breached compared to other nitrogen sources in producing GABA for *Pediococcus acidilactici* DS15; however, peptone was found as the best source of nitrogen in the growth of bacteria.

Figure 2 shows that the use of organic nitrogen sources (peptone, yeast extract, tofu water, and soy milk) increased GABA production and cell growth compared to inorganic nitrogen sources (KNO_3, NH_4NO_3, and urea). This result is in line with the research of Zhao et al. (2015) that reported the use of organic and inorganic nitrogen sources lead to a difference in the yield of GABA produced by LAB. In the mentioned study, when a single inorganic nitrogen source such as urea, ammonium sulfate or citric acid diamine was given, the growth of *L. buchneri* WPZ001 was severely inhibited and the production of GABA reduced. But when peptone fish meal, meat extract, or stumped peptone were given singly, both cell growth and GABA production were higher.

Tofu liquid waste, also called whey tofu, is a by-product in the process of tofu production. Whey tofu contains organic compounds such as organic nitrogen (7.61%), total sugar (0.32%), reducing sugars (0.09%), and minerals (Ghofar et al. 2005). these organic compounds make whey an appropriate growth media for bacteria. The nutrient content of soybeans is what distinguishes whey tofu from commercial nitrogen sources including peptone, yeast extract, NH_4NO_3, and KNO_3.

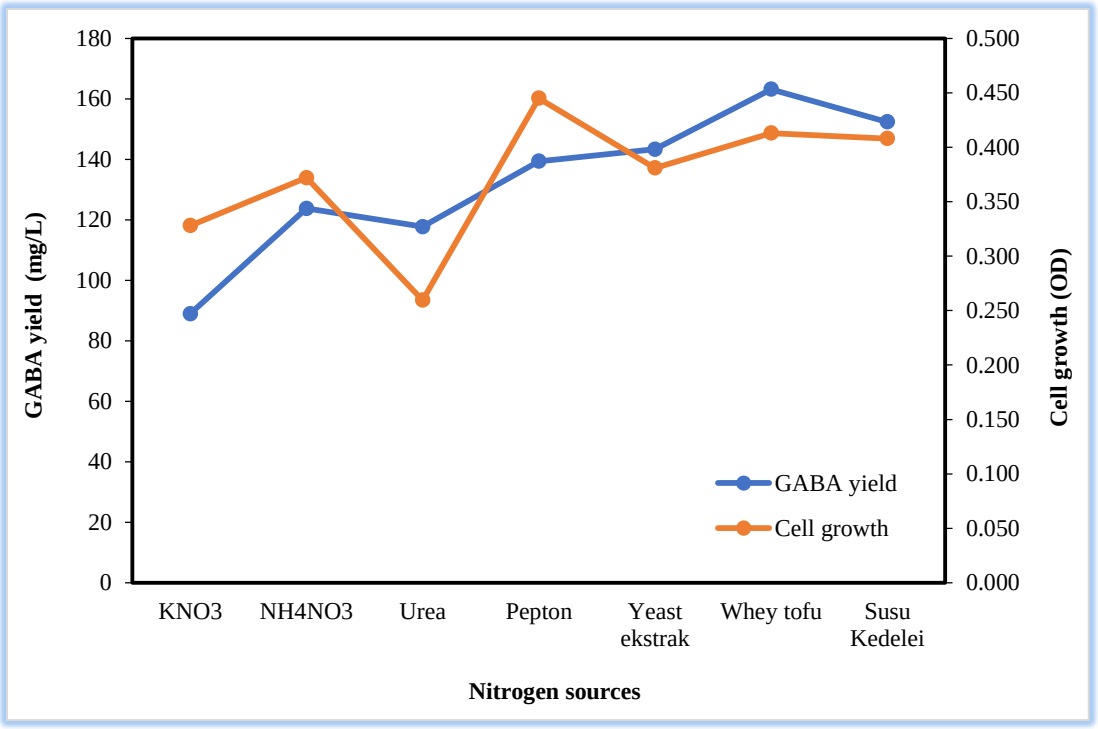

Figure 2. GABA production and growth curve of *Pediococcus acidilactici* DS15 in various nitrogen sources

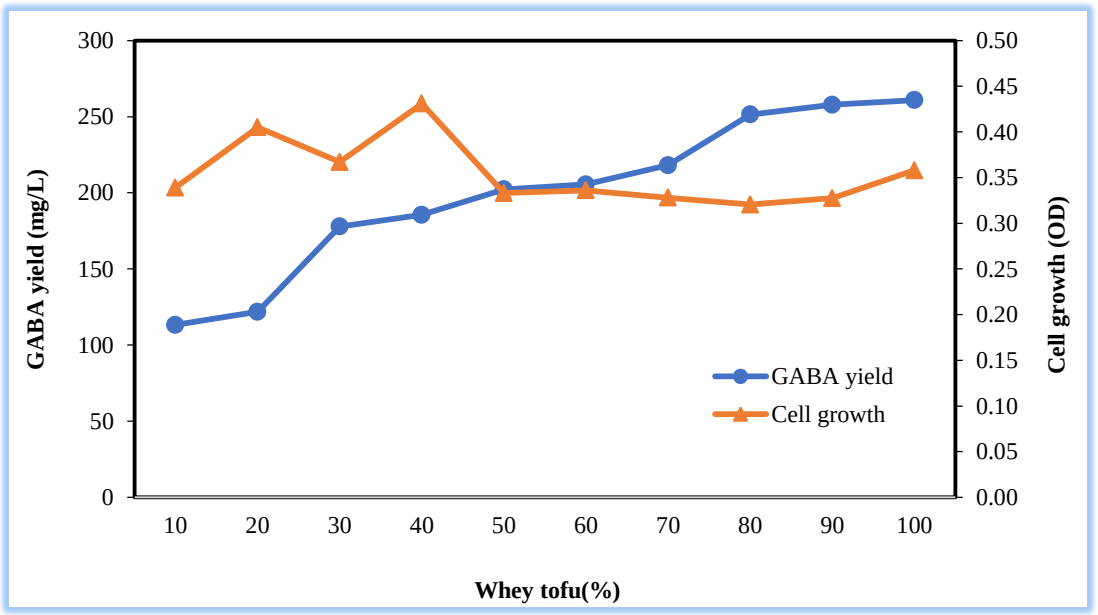

Figure 3. GABA production and growth curve of *Pediococcus acidilactici* DS15 based on the level of tofu liquid waste supply

Level of use of tofu liquid waste

The nitrogen source is used as a constituent material of cell biomass. LAB in the growth phase utilizes protein as a source of nitrogen, which is used by bacteria for protein synthesis, amino acids (Nisa et al., 2001). The highest cell growth was found in 40% of the use of whey tofu, and the use of more than 40% reduced cell growth (Figure 3). This decline is due to the high nitrogen content being the limiting factor for cells to grow. Changes in nutrient availability affect growth and biomass products (Leroy and de Vuyst, 2001).

Figure 3 shows that GABA production increased with increasing dosage of whey tofu used. This is because higher the doses of whey tofu are richer in organic compounds such as organic nitrogen and minerals. According to the obtained results, it was found that 100% use from whey tofu could provide a good source of nitrogen for *Pediococcus acidilactici* DS15 to produce GABA.

Utilization of carbon sources

In the present study, several types of carbon sources in the form of simple sugars were used to determine the effects of type and amount of carbon sources on GABA production by *Pediococcus acidilactici* DS15.

Figure 4 illustrates that palm sugar is the best source of carbon compared to glucose, maltose, cane sugar, and sucrose which were used for the GABA production. In contrast to GABA production, the graph of cell growth of *Pediococcus acidilactici* DS15 showed that the highest cell growth was found in glucose as a carbon source, amounting to 0.419, while the lowest was 0.378 in maltose. This difference can indicate that there is no correlation between the level of production of GABA and the number of bacterial cells.

The highest GABA production by *Pediococcus acidilactici* DS15 was 140.6 mg/L in utilizing palm sugar, followed by glucose, sugar cane, lactose, maltose, and sucrose with a production of 115,774; 110.2; 109,554; 94,284 and 79,813 mg/L, respectively. In contrast to the study of Soe et al. (2013) which used *Lactobacillus brevis* to produce GABA, it was found that sucrose was the best source of carbon compared to fructose and maltose, which was 23.64 mM, while other studies reported that maltose is the best carbon source in GABA production by *L.brevis* K203 (Binh et al., 2014) and *L.brevis* HYE1 (Lim et al., 2017). In addition, xylose was described as the best carbon source *L. buchneri* WPZ001 to product GABA (Zhao et al., 2014). GABA production is affected by differences in the types and strains of LAB because each strain of LAB has differences in the use of carbon sources that can have impacts on growth and function of bacteria.

The cell growth of the *Pediococcus acidilactici* DS15 revealed differences in various carbon source. The highest growth is indicated by glucose, followed by sucrose, lactose, palm sugar, cane sugar and maltose. This difference in cell growth is caused by the type of sugar in each source. Palm sugar has a sugar content in the form of sucrose, which is a disaccharide composed by glucose and fructose, and a dextran which is a polysaccharide that has a chain of glucose branches. Glucose is usually a good source of carbon for bacterial growth but interferes with the formation of secondary metabolites (Demain, 1989). Papagianni and Sofia (2009) revealed that *Pediococcus acidilactici* can use sucrose as a carbon source. The culture medium contained a mixture of simple and complex carbon sources, the simple carbon source is used for cell formation and little or no secondary metabolites formation. Complex carbon sources will be used for idiolites formation after the simple carbon source has been used (Ruiz et al., 2010).

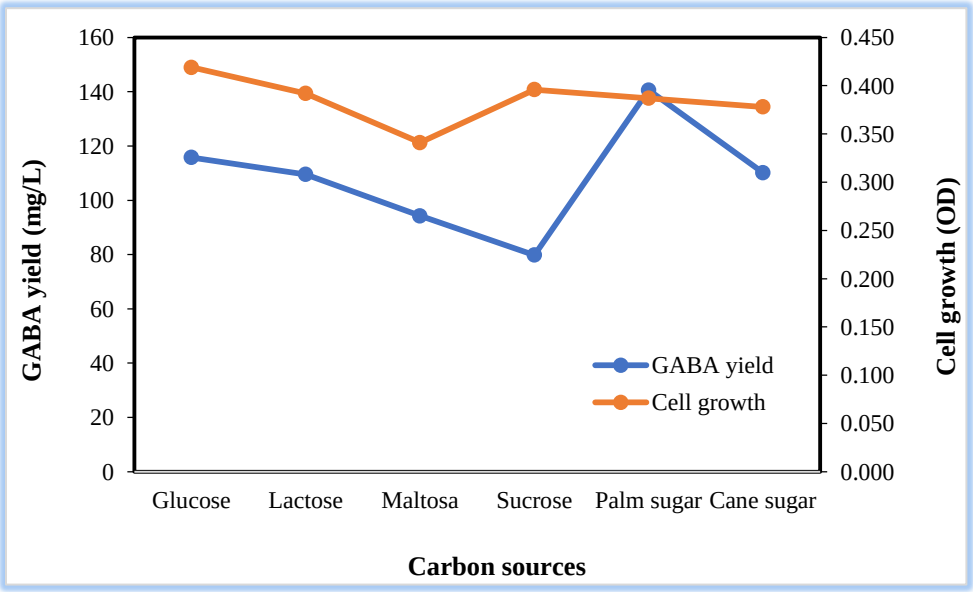

Figure 4. GABA production and growth curve of *Pediococcus acidilactici* DS15 in various carbon sources

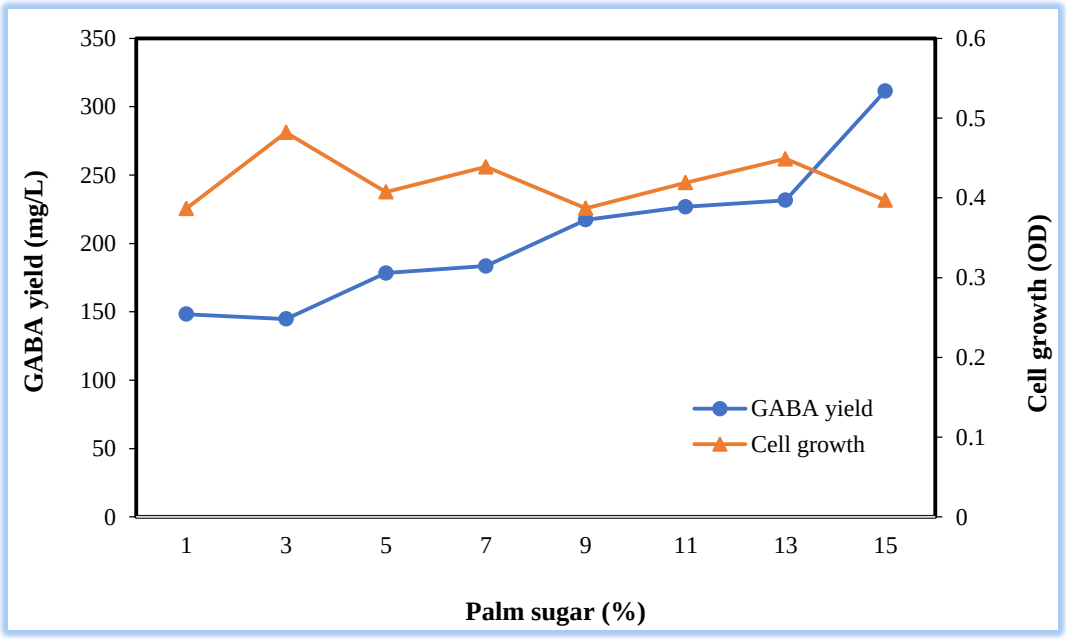

Figure 5. GABA production and growth curve of *Pediococcus acidilactici* DS15 based on the level of administration of palm sugar

Level of use of palm sugar

In the current study, the optimum dose of palm sugar as best carbon source were determined. According to the obtained results, it demonstrated that the production of GABA has increased along with the increase in the provision of palm sugar up to 15% by 311,485 mg/L (Figure 5). Previous studies have been reported that in order to GABA production, the best carbon sources to add to MRS media are 4% sucrose for *Lactobacillus sakei* B2-16 (Kook et al., 2010), 3% sucrose for *L. brevis* 340G (Soe et al., 2013), 1% glucose for *L.buchneri* MS (Cho et al., 2007).

Palm sugar has high sucrose content which is used by *Pediococcus acidilactici* as an energy source. Addition of sucrose can increase the number of carbon sources as an energy source for cell growth so that the production of primary and secondary metabolites will increase.

The growth of microorganism including bacteria or fungi was strongly influenced by the presence of sufficient carbon sources, optimal temperatures, suitable pH conditions and other supporting conditions. Carbon sources that act as nutrients are needed for the survival of bacteria in producing primary metabolites as a necessity of life. If the nutrients contained in the media are overgrown in abundant amounts, then cell viability will increase

CONCLUSION

The best source of nitrogen and carbon in producing GABA by *Pediococcus acidilac*tici DS15 were tofu water and palm sugar, respectively. The best concentration was 100% tofu liquid waste and 15% palm sugar resulted in production rate of GABA up to 311,485 mg / L.

DECLARATIONS

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Author's contribution

Lili Anggrani and Yetti Marlida conducted the research, prepared data and wrote the article. Wizna, Jamsari and Mirzah cheked and confirmed the final form of article.

Competing interests

The authors declare that they have no competing interests.

Consent to publish

All the authors gave their informed consent prior to their inclusion in the study.

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Fermentation of *Sargassum binderi* Seaweed for Lowering Alginate Content of Feed in Laying Hens

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ABSTRACT

The object of this study was to reduce the alginate content of *Sargassum binderi* by fermentation of *Bacillus megaterium* S245 in feed of laying hens. The experiment was arranged in a factorial with completely randomized design. The first factor was different inoculum dosage (1, 3, 5, 7, and 9%), while the second factor was different fermentation period (1, 3, 5, 7 and 9 days), and each treatment was replicated for five times. The measurements were total dry matter, organic matter, ash, alginate, and crude protein. The results showed there were no significant effect of inoculum dosage, and interaction between inoculum dosage and fermentation period on alginate, total dry matter, organic matter, ash and crude protein content of *Sargassum binderi* while fermentation period reduced the alginate and total dry matter content. Fermentation period also increased the crude protein content significantly. Besides that, fermentation period didn't effect on organic matter and ash content significantly. The fermentation of *Sargassum binderi* with *Bacillus megaterium* S245 at inoculum dosage of 1% and fermentation period of nine days was the best combination for lowering alginate content in *Sargassum binderi* and this treatment had positive effect on nutrient content of *Sargassum binderi*.

Key words: Alginate, *Bacillus megaterium S245*, Fermentation, Laying hens, *Sargassum binderi*

INTRODUCTION

Brown seaweed (*Phaeophyceae*) is one of the seaweed divisions that has variety of species, such as *Ascophyllum*, *Durvillaea*, *Ecklonia*, *Laminaria*, *Lessonia*, *Macrocystis*, and *Sargassum* (Mc Haugh, 2003). Mc Haugh (2003) explained that brown seaweed strain *Ascophyllum* is founded in seas of Scotland, Iceland, Norway, and Canada, and strain *Durvillaea* is founded in seas of Tasmania and Australia, while strain *Ecklonia* is founded in seas of South Africa South Korea. The brown seaweed strain *Laminaria* is founded in seas at France, Norway, Scotland, and Iceland, and brown seaweed *Lessonia* is founded in seas of Chile, while brown seaweed strain *Macrocystis* is founded in seas of United States of America, Mexico, Chile, Argentina, and South Africa, and brown seaweed strain *Sargassum*, *Turbinaria*, and *Padina* are widely found and quite abundant in Indonesian seas (Rachmaniar, 2005).

Seaweed is a marine resource that has the potential to be developed as a nonconventional feed for poultry. They are considered the most important food supplement of the 21century as a source of proteins, lipids, polysaccharides, minerals, vitamins, and enzymes (Rimber, 2007). According to Rasyid (2004), seaweed can be used as a mixture of animal feed, especially in maritime countries. Now the availability of seaweed as animal feed increased with the production of seaweed feed in form of powder seaweed (Mc Haugh, 2003).

Sargassum binderi is species of brown seaweed (*Phaeophyceae*). *Sargassum binderi* contains crude protein 6.93%, alginate 20.89% (Dewi et al., 2018), crude fat 1.07%, crude fiber 7.76%, metabolism energy 2179.63 kcal/ kg, Ca (Calsium) 0.64%, and P (Phospor) 0.62% (Analysis of Nutrition of Non Ruminant Laboratory, 2019). Brown seaweed contain alginate (Dewi et al., 2018), fukoidan (Synytsya et al., 2010; Song et al., 2012), fucoxanthin (Haugan et al., 1995; Matsuno, 2001), and

unsaturated fatty acid (PUFA/Poly-Unsaturated Fatty Acid) (Al-Harthi and El-Deek, 2012a; Carrillo et al., 2012; Pal et al., 2014). This bioactive compound has hypocholesterolemic activity, antiviral, antibiotic, anti-inflammatory, antithrombin, anticoagulation, antilipemic, and stimulant (Al-Harthi and El-Deek, 2012a). Based on previous research showed that cholesterol in yolk was lowered by alginate and fucoidan compounds in brown seaweed (Carrillo et al., 2012). In addition, alginate as water soluble fiber can lower cholesterol in the blood especially LDL (Low Density Protein) and act as antihyperlipidemic compound (Mao et al., 2004). Furthermore, Al-Harthi and El-Deek (2012b) reported fucoxanthin may reduce cholesterol, but it is also known to increase pigmentation in egg yolks. Fatty acids in seaweed are reported that have a role in reducing the cholesterol level in egg yolks (Carrillo et al., 2012).

Sargassum binderi contains high salt, it reaches 17.20% (Dewi et al., 2018). The immersion of Sargassum binderi in water flow was the best method for lowering high salt content in Sargassum binderi. Dewi et al. (2018) reported, the immersion of Sargassum binderi for 15 hours in water flow could reduce salt content in Sargassum binderi from 16.86% to 0.94%. However, this method has not been able to reduce dominant carbohydrates (alginate) in seaweed. Alginate is a family of linear binary copolymer of β-1,4 -D-manuronic acid and β-1,4-L-guluronic acid residue and contained widely varied composition and sequence (Draget, 2009). Alginate is a polyuronic saccharide that is isolated from the cell walls of a number of brown seaweed species around the world, and it is also produced as an extracellular matrix by certain bacteria (Stokke et al., 2000; Draget et al., 2005; Draget, 2009; Nalamothu et al., 2014). Alginate compound reach up 40% from the dry matter of seaweed (Draget et al., 2005). This high alginate content in the poultry feed will bind nutrients and inhibit absorption in the gastrointestinal tract, so that it will interfere performance of poultry (Riski, 2015; Hendro, 2015; Zulhaqqi, 2015; Zahara, 2015). On the other hand, proper concentration of alginate in poultry ration will help for lowering fat digestion by binding fat in the digestive tract, and then alginate with undigested fat will excreted as feces (Kasahara et al., 2018). Mushoilaeni et al. (2015) reported, that feeding of alginate to mice in hypercholesterolemia conditions as much as 0.75-1% lowering cholesterol in blood serum of mice by 53%.

Feed stuff fermentation process with microorganism can improve quality of feedstuff nutrient, digested, and elongate of feedstuff storage.

According to Rizal et al. (2013) palm kernel cake fermented with *Aspergillus niger* improve the nutrient content and nutritional quality. Crude fiber in pineapple peel waste was reduced by fermentation using cellulolytic local microorganism solution derived from bamboo sprouts (Adrizal et al., 2017). Heryandi et al. (2018) reported fermented of pineapple peel waste could be used in broiler diet up to 12% without negative effects on organ development and carcass performance.

Subaryono et al. (2016) reported, bacterium *Bacillus megaterium* S245 produce alginate lyase This bacterium includes of negative gram bacterium which was isolated from *Sargassum crassifolium* brown seaweed. Furthermore, it is explained this enzyme can depolymerize polymanuronate or polyguluronate from alginate structure, this bacterium also was used to produce oligosaccharides alginate from alginate (Subaryono et al., 2016). So far, there is no report regarding the utilization of *Bacillus megaterium* S245 as inoculum to ferment *Sargassum binderi* brown seaweed for lowering its alginate content thought fermentation method, in feed of laying hens. We are already performed the experiment for lowering alginate in *Sargassum binderi* by using *Bacillus megaterium* S245 as inoculum.

MATERIALS AND METHODS

Collection of *Sargassum binderi* seaweed

Sargassum binderi seaweed was collected by a simple random sampling method at Nipah Beach, Pesisir Selatan District, West Sumatra, Indonesia. Whole individuals of *Sargassum binderi* were used in this experiment.

Fermentation of *Sargassum binderi* with *Bacillus megaterium* S245

The fermentation process in this study was liquid fermentation type. The total substrate (consist of *Sargassum binderi* seaweed flour, palm sugar, and water) in this experiment was 300 ml, and the ratio of *Sargassum binderi* seaweed flour with water was 1: 5, and palm sugar was added 3% from the total volume of substrate. The levels of *Bacillus megaterium* S245 as inoculum were 3, 6, and 9% from the substrate. Fermentation period was 1, 3, 5, 7, and 9 days for each treatment. This experiment was performed in a factorial completely randomized design. The first factor was inoculum dosage of *Bacillus megaterium* S245 consist of 3, 6, and 9%, and the second factor was fermentation period was 1, 3, 5, 7, and 9 days, and each treatment was replicated for five times.

Preparation of *Sargassum binderi* samples as product of fermentation for analysis

After fermentation, the *Sargassum binderi* seaweed was dried in oven, then crushed to a dry powder with blender (Philips). Furthermore, samples were analyzed for alginate by Zaelanie et al. (2001) method, total dry matter, organic matter, ash, and crude protein content of *Sargassum binderi* seaweed by AOAC (1990) method.

Analysis of content

Alginate was analyzed by Zaelani et al. (2001) method. One g of *Sargassum binderi* seaweed flour was soaked in 10 ml of HCl 0.5% for 30 minutes, and then, it soaked in 10 ml of 0.5% NaOH for 30 minutes. Furthermore, sample was extracted with 10 ml Na_2CO_3 7.5% at a temperature of 50 ° C for two hours in a water bath. The sample filtered, and then the filtrate was precipitated by adding 10 ml of 5% HCl, 10 ml of NaOCl to oxidize the pigments of seaweed. The gel formed was separated by centrifuge for 15 minutes with speed 3500 rpm. Furthermore, the gel precipitate was dissolved with 10ml of 5% NaOH to convert alginate acid to alginate salt, after that, it was precipitated again with 95% isopropanol solution to form alginate salts. The obtained precipitate is dried at a temperature of 60°C and weighed with a digital scale to a constant weight. Crude protein, dry matter, organic matter, and ash were analyzed by proximate analysis (AOAC, 1990).

Statistical analysis

Data were in a factorial completely randomized design and statistically analyzed via ANOVA test. The difference among treatment means was determined using the Duncan Multiple Range Test (DMRT) ($P<0.05$) (Steel and Torrie, 1980).

RESULT AND DISCUSSION

The result of fermentation of *Sargassum binderi* with *Bacillus megaterium* S245 on total dry matter, organic matter, ash, alginate, and crude protein is presented in table 1. The different of inoculum dosage of *Bacillus megaterium* S245 (3, 6, and 9%), and interaction between inoculum dosage with fermentation period (1, 3, 5, 7, and 9 days) did not affect ($P>0.05$) on alginate, total dry matter, organic matter, ash, and crude protein, while fermentation period affected ($P<0.05$) the alginate and crude protein of *Sargassum binderi*. Ash and organic matter did not affect ($P>0.05$) by inoculum dosage, fermentation period, and interaction between dosage inoculum, and fermentation period significantly.

The effect of increasing of inoculum dosage in fermentation process in this experiment did not increase of microbes ability to degrade dry matter of seaweed. It was expected that a high inoculum dosage should accelerate fermentation process, because large number of microbes will highly produce enzymes, and some dry matter will be degraded. In fact, present study found the increasing of inoculum dosage from 3 to 6 and 9% in fermentation process did not affect degradation of dry matter, because the increasing of inoculum dosage, increasing the bacteria mass which caused by high competition to obtain nutrients for growth, so that the availability of substrate nutrient decreased faster in the fermentation process in comparing with fermentation process with lower inoculum dosage (3%). According to Maier (2009), microbial growth depends on the availability of substrate nutrition, if nutrient availability of the substrate decreases, the microbial growth rate decreases. The microbial growth consist of four phases are lag phase, exponential, stationary, and dead phase, and at stationary phase showed microbial growth and dead microbial is balance. Maier (2009), explained that there are several reasons why microbes reach the stationary phase, that are decrease of substrate nutrition because it has been used by microbes to growth, and accumulation of products produced by microbes was maximum where these products inhibit microbial growth. Liu et al. (2012), also explained that the seaweed waste could not be fully decomposed by microorganisms with little microbial agents, otherwise too many microbial agents competed and did not conductive to fermentation of seaweed waste. Therefore, inoculum dosage at 6 and 9% were similar with inoculum dosage 3% to degrade dry matter of *Sargassum binderi*. According to Hardini (2010), microbes need substrate as source of carbon, nitrogen, and minerals.

Total dry matter of *Sargassum binderi* after fermented with fermentation period at 5, 7 and 9 days lower than fermentation period at 1 and 3 days. It means some compound in *Sargassum binderi* fermented such as alginate was degraded in fermentation process. Prolong of fermentation period to 5 days showed increasing dry matter degradation, but prolong of fermentation period to 7 and 9 days, the activity of alginate lyase enzyme that produced by *Bacillus megaterium* S245 in fermentation process did not increase to degrade dry matter. In accordance with Correa and Villena (2010), prolong of incubation period increase growth of microbes until the stationary phase is reached.

Table 1. Effect fermentation of *Sargassum binderi* with *Bacillus megaterium* S245 on total dry matter, organic matter, ash, alginate and crude protein

Treatment		Total Dry Matter (%)	Organic Matter (%)	Ash (%)	Alginate (%)	Crude Protein (%)
Inoculum Dosage (ID) (%)						
3		81.76	82.38	17.62	34.47	12.04
6		81.88	82.12	17.88	34.66	12.09
9		81.87	82.06	17.94	34.65	12.13
Fermentation Period (FP) (days)						
1		83.98[a]	82.42	17.58	36.39[a]	11.62[c]
3		82.13[b]	82.03	17.97	34.85[a]	12.10[b]
5		81.38[bc]	81.75	18.25	34.82[a]	12.16[ab]
7		81.31[bc]	82.16	17.84	34.76[a]	12.10[b]
9		80.41[c]	82.55	17.45	32.13[b]	12.44[a]
Interaction between Inoculum Concentrations (IC) and Fermentation Period (FP)						
IC (%)	**FP (days)**					
3	1	84.83	83.00	17.00	35.91	11.44
	3	82.10	82.42	17.58	34.78	11.88
	5	81.15	81.58	18.42	34.62	12.25
	7	80.54	82.42	17.58	35.24	12.00
	9	80.20	82.46	17.54	31.77	12.62
6	1	83.08	82.46	17.54	36.02	11.58
	3	81.48	82.10	17.90	34.19	12.37
	5	81.61	81.78	18.22	35.01	12.16
	7	82.77	81.48	18.52	35.81	12.04
	9	80.45	82.76	17.24	32.61	12.30
9	1	84.02	81.82	18.18	37.23	11.83
	3	82.79	81.57	18.43	35.58	12.06
	5	81.38	81.89	18.11	34.84	12.07
	7	80.62	82.59	17.41	33.22	12.26
	9	80.56	82.44	17.56	32.36	12.41
Analysis of variance						
ID		ns	ns	ns	ns	ns
FP		0.05	0.05	0.05	0.05	0.05
ID × FP		ns	ns	ns	ns	ns

[a,b,c]Means in a column under similar treatment not sharing the same superscript are significantly different at (P<0.05); IC: Inoculum Concentrations; FP: Fermentation Period; ns: not significant; ID: Inoculum Dosage

Nutrient composition in *Sargassum binderi* changed along fermentation period such as reducing of alginate, and increasing of crude protein. In this experiment fermentation process did not affect on ash and organic matter content in *Sargassum binderi*. Ash content in fermented *Sargassum binderi* increased after fermented for all fermentation period (1, 3, 5, 7, and 9 days), compared with ash content in unfermented *Sargassum binderi*, while organic matter content in *Sargassum binderi* for all fermentation period (1, 3, 5, 7, and 9 days) was decrease compared with organic matter in unfermented *Sargassum binderi.* According to Ardiansyah et al. (2018) fermentation of *Sargassum flour* with *Aspergillus niger*, *Saccharomyces cerevisiae*, and *Lactobacillus* spp increased ash content. Aslamyah et al. (2017) reported that fermentation of *Kappaphycus alvarezii* green strain, *K. alvarezii* brown strain, *Gracilaria gigas*, *Sargassum sp*., and *Caulerpa sp* could decreased ash content of seaweed. It is reported the ash content increased in fermented Sargassum due to contribution of fermentation microorganisms in the degradation of organic components during fermentation (Oseni and Ekperigin, 2007). Djunaidi and Nasir (2001) obtained organic matter decreasesd because it is used as an energy source for microbes**.** This result differs from research of Felix and Brindo (2014) that expressed the ash

content of fermented *Kappaphycus alvarezii* decreased as compared with unfermented *Kappaphycus alvarezii.*

Alginate is a polysaccharide group widely found in brown seaweed (*phaeophyceae*), and this carbohydrate as calculated as dry matter in analysis of food substances by the proximate analysis method. In this experiment, there is no effect of all of inoculum dosage and interaction of inoculum dosage with fermentation period on the alginate content in *Sargassum binderi*. According to Draget et al. (2005), and Draget (2009), alginate is a polysaccharide compound found in brown seaweed (*phaephyceae*), its compound is composing of salts of alginic acid with β-1,4-D-mannuronic acid and α-1, 4-L-guluronic acid bonds.

Table 1 showed, the fermentation period of S*argassum binderi* by alginate lyase which produced by *Bacillus megaterium* S245 in 1, 3, 5, and 7 days fermentation period were not enough to reduce alginate content in *Sargassum binderi*. Therefore, fermentation period on 3, 5, and 7 days did not affect the alginate ($P > 0.05$). However the extension of fermentation period to 9 days caused a significant decrease in alginate ($P<0.05$). According to Ostgaard et al. (1993), alginate from *Laminaria saccharina* brown seaweed was degraded by combination of *Ascophillum nodosum* and cow manure as much as 10% still be found after 30 days fermentation process. It is mean degradation of alginate need a long period. The best fermentation period to reduce alginate content in *Sargassum binderi* in this experiment was found at nine days with alginate content was 32.13%. According to Draget (2005), alginate lyase catalyzes the depolymerization of alginate by splitting the 1-4 glycosidic linkage in β-elimination reaction, leaving an unsaturated uronic acid on the non-reducing end of the molecules. The research by Zhang et al. (2012) and Liu et al. (2012) show that fermented from seaweed waste need long fermentation period for 15 days to improve its quality of nutrition. The result of this research was different with findings of Moen (1997) that indicated the alginate degradation of *L. hyperborea* started after 50 hours, the viscosity of the polymer rapidly decreased within the period 50-100 hours, and after 100 hours no alginates could be isolated since the extractable part was completely depolymerized.

In this experiment, the increasing of fermentation period would increase crude protein content in fermented *Sargassum binderi.* The increasing of crude protein in fermented *Sargassum binderi* was from bacterial mass and enzymes that produced by *Bacillus megaterium* S245. Bacteria mass and enzymes are proteins. According to Azhar (2016), bacterial cell wall is composed of proteins, and enzymes produced by bacteria are proteins. This result agrees with Zhang et al. (2012) that reported fermented seaweed waste could be increase of crude protein as much as 34.56%, and also Ardiansyah et al. (2018) reported that fermentation of *Sargassum flour* with *Saccharomyces cerevisiae*, and *Lactobacillus* spp increased crude protein content. Felix and Brindo (2014) reported that crude protein content of raw and fermented *Kappaphycus alvarezii* are 14% and 23.86% respectively. This result showed that increased of crude protein content of fermented *Kappaphycus alvarezii*. Furthermore, Tangendjaya (1993) explain that increase amount of microbial mass will cause increase content of fermented products, where protein content is reflection of cell mass microbe, which in fermentation process, microbe produces enzymes that will degrade complex compounds into simple material, and microbes will also synthesize proteins which are protein enrichment process, that are enrichment of crude protein.

CONCLUSION

The fermentation of *Sargassum binderi* with *Bacillus megaterium* S245 with inoculum dosage 1% and fermentation period nine days were the best combination for lowering alginate content in *Sargassum binderi* and this treatment has positive effect on nutrient content of *Sargassum binderi*.

DECLARATIONS

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Competing interests

The authors declared that they have no competing interests.

Author's contribution

Dewi wrote the paper, collected data, and performed statistical analysis. Yuniza, Nuraini, Sayuti, and Mahata created the idea and designed the study. Dewi and Mahata drafted the manuscript and approved the final manuscript.

Consent to publish

All authors informed their consent prior to inclusion in the study.

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Effect of Glutamate Supplementation as a Feed Additive on Performance of Broiler Chickens

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ABSTRACT

Feed additives are ingredients that are added to the ration as growth promoters and enhancement of the immune system. Glutamate is a feed additive that improves performance by improving the quality of the small intestine and enhancing the immune system. The purpose of this study was to know the effect of including glutamate as a feed additive in improving broiler performance. The material used in this study was broiler strain MB 202 from PT Charoen Phokphand Indonesia as many as 240 birds. The design used was a Completely Randomized Design (CRD) trial design, with six treatments and four replications, so that there were 24 cage plots as experimental units. Each experimental unit consisted of 10 chickens. The Glutamate doses in groups were, A (0.4% commercial glutamate; B (0% glutamate); C (0.2% glutamate); D (0.4% glutamate); E (0.6% glutamate); F (0.8% glutamate). The results indicated that glutamate up to 0.8% had significant effects on feed intake body weight gain and feed conversion ratio (1.70%), but it did not affect the percentage of carcass. It is concluded that including the 0.8% glutamate in broiler disets can improve broiler performance with 35 days of maintenance.

Key words: Feed additive, Glutamate, Growth promoters, Immune system, Performance

INTRODUCTION

The broiler is one of the meat-producing livestock that is quite potent in meeting the people's needs for animal protein needs. This is because broiler meat is relatively cheap and easy to obtain compared to other animal proteins. According to Ensminger et al. (2004) broilers have a fast and efficient growth in converting feed into meat. In addition, broilers also have weaknesses which tend to be susceptible to disease attacks which results in a decrease in broiler performance.

Supplementation of additional feed is one solution to prevent disease attacks by increasing immunity and growth promoters. According to Madhupriya et al. (2018) Feed additives are ingredients and add to rations to improve animal immunity and performance. Antibiotic Growth Promoter (AGP) is an antibiotic feed additive added to broiler feed. However, the use of AGP in broiler feed is prohibited because residues in broiler meat are harmful to public health. Residual hazards could reduce human resistance to consumption of certain types of antibiotics and other hazards (Ruegg, 2013; Singh et al., 2014). This had led to the enactment of the World Health Organization's regulation regarding the prohibition on the use of antibiotics in animal feed, hence the use of antibiotics as feed additives in broiler feed. So it is necessary to look for additional feed ingredients that are safer for livestock and the community. One safe and harmless feed additive is glutamate.

Glutamate is a feed additive that can improve growth promotes and enhances the broiler immune system. Glutamate functions was known as a constituent of proteins, a substrate in the synthesis of amino acids, as a precursor to several non-essential amino acids and helped the metabolism (Young and Ajami, 2000). According to Newholms et al. (2003); Reeds et al. (2000) glutamate could improve intestinal conditions by increasing intestinal villi length and increasing maintenance of

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intestinal integrity. In addition, according to Li et al. (2007) glutamate regulated Inductible Nitric Oxide Synthase (INOS) in certain tissues. The expression of INOS was considered as a fundamental mechanism in the protection of parasites, bacteria, fungi, malignant cells, intracellular protozoa and viruses in different animal species, including mammals and birds. The present study aimed to know the effect of giving glutamate as a feed additive in broilers.

MATERIALS AND METHODS

Place of study

This research was conducted on May-September 2018. Maintenance of broilers was conducted at the Poultry Division Field Laboratory, Faculty of Animal Husbandry, Andalas university, Indonesia.

Materials

A total of 240 one-day old male broiler chicks (MB 202 from) were purchased from PT Charoen Pokphand, Indonesia. The chickens were placed randomly into 24 cage plots (open cages), each measuring 1×1 m^2. Each plot was filled with 10 chickens, equipped with food and drinking water. This broiler cage was also equipped with 1 bulb (35 Watt). The ration was prepared by itself from feed ingredients consisting of corn, commercial feed (CP511 PT, Charoen Pokphand Indonesia), fish meal, soy flour, bran, palm oil and mineral premix. Nutrient content and feed composition was indicated in tables 1 and 2.

Table 1. Nutrient content of feed ingredients for broiler chickens

Feed ingredients	Nutritional content (%) and energy metabolism (Kkal/kg)					
	Crude Protein	Crude Fiber	Crude Fat	Ca	P	ME
Corn	9.55	3.8	2.18	0.38	0.33	3300
Bran	10.6	10.84	4.09	0.7	0.09	1592
Fish flour	41	2.8	1.52	5.55	2.6	2580
Commercial Feed*	23	1.88	5.87	0.29	0.15	3200
Soybean Meal	40.16	3.58	1.37	0.63	0.32	2240
Coconut oil	0	0	100	0	0	8600
Mineral Premix	0	0	0	5.38	1.14	0

ME: Energy metabolism, Ca: Calcium, P: Phosphor; *Commercial Feed: CP511 PT, Charoen Pokphand Indonesia

Table 2. The composition of ration in broiler chickens

Feed ingredients	Percentage of ration (%) and nutrient content (%)
Corn	40
Bran	7
Fish flour	17
Commercial Feed*	20
Soybean Meal	14
Coconut oil	1.5
Mineral Premix**	0.5
Total	100
Crude Protein	21.75
Crude Fiber	3.86
Crude Fat	4.12
Ca	1.50
P	0.83
ME (Kcal/kg)	2952,64

Ca: Calcium, P: Phosphor, ME: Energy metabolism; *Commercial feed: CP511 PT, Charoen Pokphand, Indonesia; **Mineral Premix: Supplemented for kg of the diets: Vit. A, 12000 IU; D3, 2000 IU; E, 20 mg; K3, 3 mg; B2, 7 mg; B3, 12 mg; B5, 3 mg; B12, 0.03 mg; biotin, 0.1 mg; choline chloride, 300 mg; Mn, 130 mg; Fe, 70 mg; Zn, 60 mg; Cu,12 mg; I,1 mg; Se, 0.2 mg, and adequate antioxidant.

Method

Making glutamate was done by fermentation with *Lactobacillus plantarum*. The nutritional composition of fermentation media used in this study was KH2PO4, MgSO4 _ 7H2O, FeSO4 _ 7H2O, MnSO4 _ 4-5H2O, 9% sugar cane, 5 µ / L biotin, 90% tofu water, 10% distilled water, water starter and starter 9%. The duration of fermentation in this study was 36 hours at 36°C. After fermentation the media were centrifuged for 20 minutes and 10,000 rpm at a temperature of -4°C. After that, the supernatant was concentrated using an evaporation technique in an oven with a temperature of 40°C for 48 hours. The shrinking material was then calculated for the acid content using glutamate HPLC (Maslami et al., 2018).

Maintenance of broilers

Maintenance was carried out for five weeks. Feed and drinking water were available ad libitum. Glutamate treatment was given to chickens aged two weeks to six weeks. Glutamate was added to drinking water. The body weight was carried out at the beginning of the study and every following week, and also at the end of the study. Weighing the rest of the feed was done every weekend. Calculations were carried out for Feed Intake (FI), Body Weight Gain (BWG), Feed Conversion Ratio (FCR) and Carcass Percentage (CP).

Experiment and data analysis

This study used a completely randomized design. Each treatment was repeated four times. Each treatment and each replication consisted of 10 birds. The observed variables were just performance traits. The variable performance included average feed intake, body weight gain, feed conversion ratio and carcass percentage. The data obtained were analyzed using analysis of variance (Analysis of Variance / ANOVA) and if there were differences between treatments were tested further using the DMRT (Duncan Multiple Range Test) tests at a level of 5% (Steel and Torrie, 1991). The dose of glutamate in drinking water is A (0.4% commercial glutamate), B (0.0% glutamate), C (0.2% glutamate), D (0.4% glutamate), E (0.6% glutamate) and F (0.8% glutamate).

RESULTS AND DISCUSSION

The results indicated that giving of glutamate had a significant effect (P<0.05) on feed intake, body weight gain and feed conversion ratio. While the giving of glutamate did not have a significant effect (P>0.05) on the percentage of the carcass. The average FI, BWG, FCR and CP by giving glutamate was indicated in table 3.

Table 3. Effect of dietary inclusion of glutamate on broiler chicken performance for 35 days

Treatment	FI (g)	BWG (g)	FCR (%)	CP (%)
A (0.4% Commercial Glutamate)	$4591.13^{a} \pm 63.67$	$2688.5^{a} \pm 24.73$	$1.71^{b} \pm 0.03$	67.23 ± 3.52
B (0 % Glutamate)	$4429.05^{b} \pm 77.25$	$2391.50^{c} \pm 28.25$	$1.85^{a} \pm 0.05$	68.76 ± 1.80
C (0.2 % Glutamate)	$4478.75^{ab} \pm 84.25$	$2450.63^{c} \pm 54.96$	$1.83^{a} \pm 0.05$	73.21 ± 3.62
D (0.4 % Glutamate)	$4530.28^{ab} \pm 76.08$	$2573.60^{b} \pm 63.79$	$1.76^{b} \pm 0.07$	70.70 ± 3.44
E (0.6 % Glutamate)	$4503.70^{ab} \pm 103.94$	$2634.15^{ab} \pm 31.66$	$1.71^{b} \pm 0.04$	70.13 ± 3.04
F (0.8% Glutamate)	$4588.68^{a} \pm 26.24$	$2693.95^{a} \pm 74.06$	$1.70^{b} \pm 0.05$	70.47 ± 4.39

FI: Feed intake, BWG: body weight gain, FCR: feed conversion ratio, CP: Carcass percentage.

Feed intake

It can be seen that group B was not significantly different (P>0.05) in the treatment of C, D and E (Table 3). Judging from the average value there was a tendency to increase feed consumption with an increase in the dose of glutamic acid which can be the same as the consumption of ration by providing commercial glutamate. Increased consumption of rations with the addition of glutamate caused by glutamate can improve the quality of the digestive tract which can increase the absorption of nutrients. According to Ebadiasl (2011) glutamate in small bowel function as the formation of intestinal mucous villi cells. Bartell and Batal (2007) reported that glutamate supplementation could improve intestinal development with an increase in the relative weight of the duodenum and jejunum. Improving the development of the small intestine can increase the capacity of the digestive tract to accommodate the volume of the feed so that, many different rations can be consumed by broilers. According to Ibrahim (2008), larger intestines could increase ration consumption because a larger volume of food can be accommodated, digested, and absorbed.

In addition, the increase in ration consumption in this study was due to a decrease in stress on broilers. Reducing stress by adding glutamate because it can synthesize amino acids which have a role in reducing stress levels. According to Young and Ajami (2000) stated that glutamate is an amino acid builder protein which is a precursor for Gamma AminoButyric Acid (GABA). Glutamate will be converted into GABA if broilers were under stress. According to Wang et al. (2015) and Lener et al. (2017) GABA served to reduce stress so that it can maintain appetite and increase the efficiency of the use of body nutrients in livestock. Thus giving glutamate to broiler chickens can reduce stress by increasing feed consumption. Feed intake in this study was between 4478.75-4588.68 g lower than that obtained by Bezerra et al. (2015) which was 5303 g for 42 days of maintenance. Similar study was presented by Olubodun et al. (2015) that administrated 0.5% of glutamate to broilers and indicated consumption of 4083 g feed.

Body weight gain

The body weight gain was increased as an increasing dose of glutamate. Giving glutamate in the treatment of F can increase weight gain, but not significantly different (P>0.05) with treatment E. Body weight gain was influenced by feed consumption, the higher the consumption of food, the more weight gain was generated. According to Fadilah (2005) feed consumption had a positive effect on weight gain. Increased glutamate dose in this treatment caused by an increase in absorption of

nutrients in small intestine. This is supported by the opinion of Shakeri et al. (2014) stated that administration of glutamate in broiler feed could improve small bowel development, intestinal villi length and nutrient absorption. Glutamate in the small intestine plays a role in the mechanism of intestinal mucosal defense by increasing the mucosal layer (Akiba et al., 2009). Increasing the inner surface and surface area of the small intestine will increase the digestion and absorption of feed extracts by the small intestine (Yao et al., 2006).

Giving glutamate can increase broiler weight because glutamate is a precursor for non-essential amino acids, so it will meet the needs of several other essential amino acids. According to Blachier et al. (2009) stated that glutamate is a precursor to other non-essential amino acids, such as arginine, glutamine, and proline. With the fulfillment of the need for non-essential amino acids will increase the growth of broilers. According to Ajinamoto (2007) and Maslami et al. (2018) administration of glutamate in broiler chicken feed could increase the growth of connective tissue so that, it can increase broiler body weight gain.

The highest weight gain in this study was found in treatment F (2646.45 g / bird) which could match treatment A with commercial glutamate (2638.35 g / bird). The body weight gain in broiler chickens of this study was higher than that reported by Olubodun et al. (2005) at 42 days of age (2375 g / bird). Increased body weight gain by adding glutamate to broilers diet was reported by Porto et al. (2015). Furthermore, according to Bezerra et al. (2015) the addition of 1.76% glutamate could match the increase in broiler body weight gain without a reduction in crude protein ration. Moreover, according to this study addition of 1.76% glutamate could increase body weight gain of broilers with a crude low protein ration.

Feed conversion ratio

The results indicated that treatment groups (D, E and F) gave the same low FCR, even matching treatment A with commercial glutamate. FCR was low by giving glutamate along with increased feed intake and body weight gain. A low FCR indicates an increase in feed efficiency (Razak et al., 2016). According to Usman (2009) and Zuidhof et al. (2014) the values of FCR were influenced by the amount of feed intake and body weight gain. Another factor is the increase in feed conversion with increasing doses because glutamate can improve the nutritional quality of feed and the digestive system. It was reported by Andriyanto et al. (2015) the nutritional quality of feed could influence broiler feed conversion. Decreased FCR caused by administration of glutamate could improve the quality of the digestive system by increasing intestinal length thereby increasing absorption of feed nutrients (Olubodun et al., 2015). Increasing absorption of feed nutrients causes an increase in body weight so that it will increase the efficiency of feed use.

The lowest FCR in this study was 1.70 lower than Shakeri et al. (2014) stated that the FCR in broilers with administration of glutamate was 1.95 Similar results were conveyed by Zulkifli et al. (2016) giving 1% glutamate to broiler could reduce FCR to 1.81 Furthermore, according to Olubodun et al. (2015) that added 0.5% of the mixture of glutamate and glutamine to broiler chicken diet and reported reduce in FCR (1.78.

Carcass percentage

The results of statistical analysis indicated that the administration of glutamate did not significantly affect ($P>0.05$) on the percentage of broiler carcass. The effect of giving glutamate on carcass percentage presented in table 3. The average carcass percentage of each treatment from treatment A to treatment F was 67.23, 68.76, 73.21, 70.70, 70.13, and 70.47% respectively. The percentage of the carcass in this study was at the range of 67.23-73.21% which was supported by Resnawati (2004) who studied in dietary addition of glutamate in broiler chickens and stated the percentage of carcass between 68-71.8%. Glutamate is a building block of proteins that can increase protein synthesis in muscle tissue. Formation of protein in muscle tissue will affect the percentage of carcasses. According to Reeds et al. (2000), glutamate is one of the building blocks of amino acids from proteins that make up the body's protein.

CONCLUSION

Addition of glutamate up to 0.8% could improve the performance of broilers for 35 days of maintenance. However, administration of glutamate did not affect the percentage of the carcass. Further researches should to be conduct to indicate the ideal glutamate dose in broilers diet.

DECLARATIONS

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Author's contribution

Vebera Maslami and Yetti Marlida conducted the research, prepared data and performed statistical analysis. Mirnawati, Jamsari and Yuliaty Shafan Nur wrote the article. All authors checked and confirmed the final form of article.

Competing interests

The authors declare that they have no competing interests.

Consent to publish

All authors gave their informed consent prior to their inclusion in the study.

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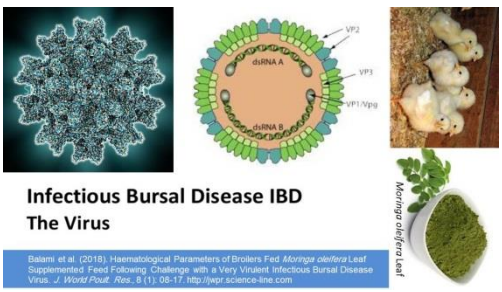

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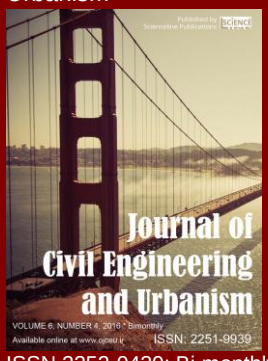

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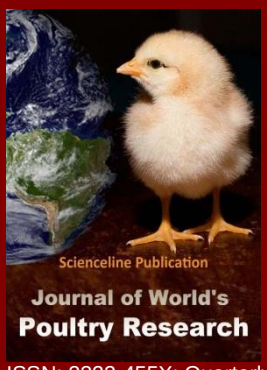

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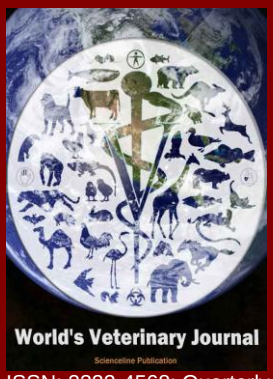

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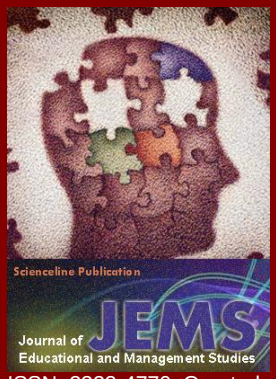

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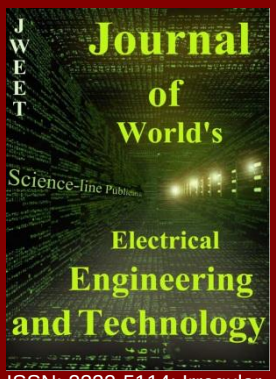

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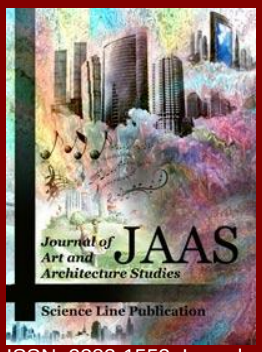

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