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Effects of *Moringa oleifera leaf* and probiotics mixed fermented extract on the egg production and cholesterol contents in egg of laying hens

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Abstract
The present study conducted to determine inclusion of drinking water supplementation with extract fermented *Moringa oleifera* by probiotics *Saccharomyces spp* (MLF) on egg production and cholesterol contents in egg of laying hens up to 70 weeks old. A total of 180 laying hens with homogenuous body weight were randomly allotted into three drinking water treatments. The dringking water treatments included: drinking without MLF extract (A); drinking water with 2% MLF extract (B); and drinking water with 2% MLF extract (C), respectively. Result showed that the MLF drinking water treatment had improved total weight eggs and egg production compared with control treatments ($P<.05$). The egg mass in MLF extract treatments B and C were greater than that in control treatment ($P<.05$). Moreover, *MSF* extract decreased significantly ($P<.05$) feed efficiencies of birds. In conclusion, fermented *Moringa oleifera* leaf by *Saccharomyces spp* extract supplementation improved egg productions and decreased cholesterol contents in eggs of laying hens.

Keywords: *Moringa oleifera*, *Saccharomyces spp*, probiotics, feed digestibility

Introduction
Antibiotic residues in animal products and bacterial resistance, causing increased concern in using antibiotics as growth promoters and finally the use of antibiotics in feed in most developed countries has been banned (Gheisar and Kim, 2018) [23]. Recently, in Indonesia there has been a ban on the use of antibiotics, so probiotics and phytochemicals of herbal leaves have been suggested as the most beneficial alternative for livestock because of their beneficial effects (Abdelqader *et al*., 2013) [1].
Supplementation of herbal plant extracts can improve taste and feed consumption, stimulate animal appetite, and then increase feed intake (Frankic *et al*., 2009) [22]. As a result, increased feed intake can contribute to an increase in poultry productivity. Different results regarding the effects of additives on herbal extracts on the performance of livestock production were reported in the studies of Gheisar and Kim (2018) [23] and Ahmed *et al.* (2017) [3]. Ahmed *et al.* (2017) [3] reported that herbal leaf supplements in natural or fermented form, apparently had no effect on growth, but reduced feed consumption and improved feed efficiency compared to controls. The fermentation process on herbal leaves can increase the efficacy of treatment of active ingredients and reduce the anti-nutritional effect on herbal leaves, thereby increasing the growth of poultry and the beneficial value of herbal leaves (Ahmed *et al*., 2017) [3]. Tannin compounds are known to have a bitter or astringent taste that can reduce palatability, thereby reducing food intake (Bidura *et al.*, 2017) [8, 9]. The fermentation process can reduce the concentration of tannic acid in herbal plants, so as to reduce the adverse effects of tannin.
The phytobiotic antioxidant function of herbal leaves positively affects food stability, improves egg quality, and extends egg storage time (eggs can be easily damaged if stored for too long). Some researchers show the positive effects of herbal phytochemical compounds on the performance of livestock (Bidura *et al*., 2017; Gheisar and Kim, 2018; Lei *et al*., 2018; Siti *et al*., 2019) [8, 9, 23, 37, 57] and probiotics (Bidura *et al*., 2019; Tufarelli *et al*., 2017; Phuoc and Jamikorn, 2017; Mountzouris *et al.*, 2010) [11, 63, 47, 42]. However, some conflicting research results regarding the performance response of livestock productivity to natural herbs, fermentation processes, or herbal leaf extraction, can be caused by different herbal species, herbal concentrations, and herbal processing methods (Windisch *et al.,* 2008; Embuscado 2015) [66, 20].

In many countries, various types of herbal plant extracts have been used in traditional medical systems to treat various types of bacterial diseases. This is due to phytochemical compounds contained in herbal plants, such as *Moringa oleifera* leaves containing: flavonoid compounds, saponins, tannins, and several other phenolic compounds that have antimicrobial activity (Bukar *et al.,* 2010; Siti *et al*., 2019) [13, 57]. Phytochemicals in *Moringa* plants that have antimicrobial and antioxidant properties are the reasons for using them in feed supplements to replace antibiotic use (Akinmoladun *et al*., 2007; Oriabi, 2016; Prasad and Ganguly, 2012) [4, 45, 49]. The antimicrobial activity of some phytochemicals in the *Moringa* plant has been investigated and the possibility of using it to develop new antimicrobial drugs (Dalukdeniya *et al*., 2016; Elangovan *et al*., 2014; Goel, 2013) [17, 19, 24]. Several previous studies confirm that extracts or compounds isolated from *M. oleifera* have antioxidant, anti-carcinogenic, anti-diabetic, anti-inflammatory, and anti-hypertensive properties, and the ability to protect against liver damage (Ashok *et al*., 2014; Chukwuebuka, 2015; Dalukdeniya *et al*., 2016; Elangovan *et al*., 2014; Godinez-Oviedo *et al.*, 2016) [6, 16, 17, 19, 25].

Likewise, yeast *Saccharomyces sp* has long been used as a "tape" fermentation inoculant, and can act as a probiotic to improve feed digestibility and poultry performance, and reduce ammonia gas content in cages (Bidura *et al*., 2012; 2019 and Ezema and Eze, 2015) [12, 11, 21]. Supplementation of fermented feed products in animal feed, can generally improve the ecology of bacteria in the digestive tract and immune response in chicks. Therefore, it becomes a new model for future strategies to control chicken disease by using herbal leaf fermentation products (Hasan *et al*., 2016; Hasanuddin *et al*., 2017; Zhen *et al.*, 2019) [27, 28, 67].

It is interesting to study the use of phytochemical herbal compounds combined with probiotics. Phytochemical and probiotic compounds, both of which exhibit antimicrobial, antioxidant, anti-inflammatory effects, and promote growth and improve feed efficiency. Therefore this study was conducted to examine the use of *Moringa oleifera* leaf extract fermented by *Saccharomyces spp.* probiotics in drinking water to increase egg production and reduce egg yolk cholesterol content in chickens.

Material and Methods

Experimental design, animals, housing and diets

A total of 180 laying hens with an average initial body weight of 1778.36±20.72 g were randomly allotted into three drinking water treatments. The dringking water treatments included: drinking without MLF extract (A); drinking water with 2% MLF extract (B); and drinking water with 2% MLF extract (C), respectively. All hens were housed in an environmentally controlled room with forced ventilation. Laying hens were provided with free access to drinking water and feed throughout experimental period. All chickens were given commercial feed specific for laying hens containing 2.750 kcal/kg of Metabolizable Energy (ME); 18% of Crude Protein; 3.5% of Calcium; and 0.45% available phosphor.

Each treatment consisted of six replicate pens with 10 birds were randomly assigned to each pen at 150×70×45 cm (length×width×height). Each experimental diet was in mash form and the birds had free access to feed and water throughout the experiment.

Preparation of natural and fermented herbs

The leaves of *Moringa oleifera* leaf are washed, dried in air and powdered. For solid fermentation, *Moringa oleifera* leaf powder is mixed with *Saccharomyces spp* at $3.7x10^7$ colony forming units (cfu)/g. After that, the mixture is immersed in distilled water to maintain a moisture concentration of 35%. The hydrated herb is then fermented at 37 ^{0}C. After fermentation for 72 hours, the fermented sample is mixed with distilled water 1: 1 (v: v) and it is meserated for 24 hours. Then filtered to get *Moringa oleifera* leaf water extract which has been fermented with *Saccharomyces spp* as a probiotic source. This water extract is then referred to as MLF extract.

Live performance

Continuous lighting and access to feed and water was provided throughout the experiment. The birds were weighed at the commencement (70 weeks of age) and the end (78 weeks of age) of the experiment. Eggs were collected daily and egg production was expressed on a hen-day basis (hen-day productions). Individual egg weights were recorded then used to calculate mean egg weight for all experimental period. The total egg mass was calculated by multiplying egg weights by egg production. Feed consumption was measured on a cage basis (hens) every week. Daily feed consumption per bird was calculated on a cage total feed consumption basis for the entire experimental period and for the number of days in all the period. Feed conversion ratio (kg of feed/kg of eggs mass) for the all period was calculated on a cage basis from egg production, egg weight, and feed consumption.

Performance, egg quality metrics, and laboratory analysis

Eggs were collected and labeled on a daily basis at 10.00 h and 15.00 h throughout the experimental period. The percent egg production was calculated. Once every one weeks, the eggs from three consecutive days were used to measure egg weight and quality. Once every one weeks, the eggs from three consecutive days were used to measure egg weight and quality. Yolk cholesterol content was analyzed for one weeks before the end of experiment. The content of cholesterol in yolk eggs were analyzed following the Liberman-Burchard methods (Lieberman and Burchard, 1980) [36].

Statistical analysis

All data were one-way analyzed with ANOVA to determine the differences among treatments. If differences were found (P<0.05), then further analysis was performed with Duncan's multiple range test.

Results

The results showed that administration of *Moringa oleifera* leaf extract fermented by *Saccharomyces sp*. (MLF) at levels 2-4 cc/100 cc of drinking water was found to have no significant effect (P>0.05) on feed and drinking water consumption (Table 1). However, it significantly (P<0.05) increased egg weight, total egg weight, and number of eggs. However, feed efficiency (feed consumption:egg weight) increased significantly (P<0.05) compared to the control group.

The average egg weight of chickens during the study in treatments B and C, were increased: 4.25% dan 4.35% higher (P<0.05) than controls. Likewise, the total egg weight of treatments B and C, were increased significantly different (P<0.05) higher: 8.23% dan 7.53% than controls.

Supplementation of fermented *Moringa oleifera* leaf (MLF) extract at levels 2-4 cc/100 cc of drinking water, can cause an increase in the number of eggs in chickens B and C, namely 3.80% dan 3.05% significantly ($P<0.05$) higher than control.
The value of *feed conversion ratio* (FCR) is the ratio between feed consumption and egg weight. The FCR value during the study in control chickens (A) was 2.43/head (Table 5.1). The mean FCR in chicken treatments B and C, were 8.23% dan 6.58% significantly different ($P<0.05$) lower than controls (treatment A).

Table 1: The inclusion of drinking water supplementation with extract fermented *Moringa oleifera* by probiotics *Saccharomyces spp* (MLF) on egg production and cholesterol contents in yolk of laying hens up to 70 weeks old.

Variabel	Perlakuan[1)]			SEM[2)]
	A	B	C	
Feed consumption (g/head/56 days)	6630.0a	6611.8a	6672.5a	39.298
Water consumption (ml/head/56 days)	16.851a	17.145a	17.291a	0.273
Egg weight (g/head)	66.65b[3)]	69.48a	69.55a	0.142
The numer of eggs (egg/56 days)	41.04b	42.6a	42.29a	0.287
Total Egg weight (g/head/56 days)	2734.93b	2960.15a	2940.82a	22.227
Feed conversion ratio (feed consumption:total egg weight)	2.43a	2.23b	2.27b	0.018
Yolk colour (1-15)	8.63b	10.12a	10.29a	0.232
Yolk cholesterol (mg/dl)	174.71a	158.32b	159.12b	1.468

Notes
1. A: drinking water without MLF as control; (B): drinking water with 2 cc/100 cc MLF extract; and (C): drinking water with 4 cc/100 cc MLF extract, respectively.
2. SEM: Standard error of treatment means
3. Means with different superscripts within raw values are significantly different ($P<0.05$)

The color value of the yolk of chicken treatment B and C increased significantly ($P<0.05$), namely 17.27% and 19.24% higher than the control. On the contrary, there was a decrease in egg yolk cholesterol content in treatment B and C chickens, namely 9.38% and 8.92% were significantly ($P<0.05$) lower than controls.

Discussion

The consumption of rations and drinking water for 56 days of research on the treatment of *Moringa* leaf water extract fermented by yeast *Saccharomyces sp.* (MLF) through drinking water did not show any significant difference with control (A). This is because the metabolic energy content of feed and nutrients for the three treatments is the same. The same thing was reported by Bidura *et al*. (2012) [12] that in general, chickens consume food to meet energy needs. If the energy needs of the chicken body are sufficient, the chicken will stop consuming feed even though the cache is still able to accommodate the feed. Feed consumption is directly proportional to the consumption of drinking water, because drinking water is very necessary to dissolve food substances. The results of this study were supported by Ekayuni *et al*. (2017) [18] who reported that herbal extracts did not affect the consumption of feed and drinking water.
Also reported by Sanchez *et al*. (2005) [52] that herbal extracts does not have toxic effects or contain factors that limit intake as opposed to nutrient absorption. Ahmed *et al.* (2017) [3] found herbal leaf flour had no effect on feed consumption. The same thing Paguia *et al.* (2014) [46] found that *Moringa* leaf meal did not affect feed consumption. Generally, administration of herbal extracts to poultry can reduce feed consumption. Decreased feed consumption can be attributed to the presence of phytate, tannin, and saponin, in herbal leaves which all can reduce feed consumption, because of its unpleasant nature and binding nutrients (Anhwange *et al.,* 2004) [5].
MLF supplementation in chicken drinking water has a significant effect on average egg weight, total egg weight and number of eggs. According to Shivaramaiah *et al*. (2011) [55], it turns out that extracting fermented herbal products using microbes given this directly, then their survival through the process of digestion, growth in the digestive tract, and able to increase the digestibility of food substances. Different results were reported by Davis and Anderson (2002) and Kalavathy *et al*. (2009) [34] that there was no increase in egg production in laying hens supplemented with probiotic bacteria, including *Lactobacillus* and *Bacillus*. Egg production in chicken fed with bacterial supplementation and yeast probiotics did not differ significantly from control chickens aged 40 to 52 weeks (Balevi *et al.*, 2001) [7]. The opposite was reported by Laxmi *et al.* (2017) that giving fermented yeast culture to sexually mature chickens during the summer period can improve feed efficiency and egg production.
Bidura *et al*. (2014) [10] reported that the use of fermented pollard with yeast in feed could significantly increase the production of Lohmann Brown chicken eggs. Yeast *Saccharomyces sp* can significantly increase the metabolic activity of absorption of food substances in the digestive tract, so as to increase egg weight and chicken egg production (Bidura *et al.*, 2019) [11]. Likewise, the efficacy of herbal leaf extracts (*Allium sativum* and *Sauropus androgynus*) at levels 2-4% in drinking water, can significantly increase egg production and reduce yolk cholesterol content in Lohmann Brown laying hens (Bidura *et al.*, 2017) [8, 9]. Egg weight increased significantly after being given MLF extract through drinking water, which showed greater resistance to diseases that affect the digestive system of chickens (Prado *et al.*, 2016) [48], so that nutrient absorption can be optimal. As reported by Dalukdeniya *et al.*, 2016) [17], that herbal leaf extract (*Moringa oleifera*) with phytochemical compounds, is very effective in controlling gram negative bacteria rather than gram positive bacteria. The same thing was reported by Goel (2013) [24], that the antimicrobial activity of herbal plants is mainly caused by the presence of secondary metabolites.
Supplementation of MLF extract in drinking water significantly increases egg weight and feed efficiency in chickens B and C groups. This increase is due to the main action of this active ingredient is the ability to inhibit pathogenic microbes and endotoxins in the chicken intestine and increase pancreatic activity, produce metabolism and

utilization better nutrition (Windisch *et al.*, 2008; Grashorn, 2010) [66, 26]. MLF water extract can be useful for use as an effective feed supplement in laying poultry to improve feed efficiency (Akhouri *et al.*, 2013).

Increased feed efficiency was seen in chicken groups that received 2-4% MLF in drinking water, when compared with the control group. This can be seen from the total weight of heavier eggs in chickens that received MLF treatment and almost all treatments had the same feed intake. Increased feed efficiency is due to the presence of phytochemical compounds on the leaves of *Moringa* and yeast *Saccharomyces sp* which function as probiotics. Likewise, the efficacy of herbal leaf extracts (*Allium sativum* and *Sauropus androgynus*) at 2-4% level in drinking water, can significantly improve feed efficiency in laying hens (Bidura *et al.*, 2017) [8, 9]. The same study has been reported by Husain *et al*. (2017); Manafi *et al.* (2018) [40]; and Sikandar *et al.* (2017) [56] that the effect of probiotics was evident in increasing feed efficiency and growth performance of broilers.

Hernandes *et al*. (2004) [29], reported that plant extract supplements can improve the digestibility of nutrients in the digestive tract of poultry. Herbal extract (garlic) can increase pancreatic enzyme activity and microenvironment conditions for better utilization of nutrients in rats (Ramakrishna *et al.*, 2003) [50]. The presence of phytochemical compounds and high nutrient content in herbal leaf extracts will increase the absorption of nutrients for egg production. Plants are rich in various secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties. This active compound in the chicken's digestive tract can help absorb nutrients. Adibmoradi *et al.* (2006) [2] reported that herbal active compounds in garlic can increase villous height and crypto depth, and reduce epithelial thickness and number of villous cells in the duodenum, jejunum, and pile ileum. Increased villi height, and thickness of the epithelium and cup in the duodenum, jejunum and ileum will increase nutrient uptake (Nusairate, 2007) [44].

The presence of yeast *Saccharomyces sp*. which is used in the fermentation process of Moringa leaves before extracting is known as a potential feed probiotic, due to the production of extraordinary extracellular enzymes including proteases, amylases, cellulases, and lipases (Chen *et al.*, 2009; Salim *et al.*, 2013) [15, 51]. This enzyme can increase the digestibility of protein nutrients, carbohydrates, and lipids in broilers (Salim *et al.*, 2013) [51]. Zurmiati *et al*. (2017) [68] and Hasan *et al*. (2016) [27] reported that supplementation of probiotic microbes (*Lactobacillus acidophilus*) alone or in combination with *B. subtilis* could significantly increase the number of beneficial bacterial populations in the intestines, nutrient digestibility, fermentation in the cecum, feed efficiency, and growth performance in rabbits (Phuoc and Jamikorn, 2017) [47].

Supplementation of MLF water extract in drinking water can significantly increase the color of the yolk, as well as reduce yolk cholesterol content. This decrease is due to the content of alkaloid compounds in *Moringa* leaves which can reduce the activity of lipogenic enzymes and increase the excretion of bile acids in feces. In addition, the presence of beta-carotene in herbal extracts can reduce blood cholesterol levels, because it can inhibit the action of the HMG-CoA reductase enzyme which plays a role in the formation of mevalonate in the biosynthesis of cholesterol in the liver (Nuraini, 2006) [43].

The ability of beta-carotene to reduce cholesterol, is related to the hydroxy methyl glutaril-CoA enzyme (Wang and Keasling, 2002). This enzyme plays a role in the formation of mevalonates in cholesterol biosynthesis. Synthesis of cholesterol and beta-carotene together through the mevalonate pathway derived from acetyl CoA. If beta-carotene consumption is greater than saturated fatty acids, the biosynthesis process by the HMG-CoA enzyme will be directed to beta-carotene synthesis, so that saturated fatty acids are not converted to cholesterol (McGilvery and Goldstein, 1996) [41]. Mevalon is needed in the process of cholesterol synthesis by inhibiting enzymes, thereby inhibiting the formation of cholesterol (Syahruddin *et al*., 2013) [60].

Phytochemical compounds, such as flavonoids, saponins, tannins, are estrogen-like compounds that can slow down the reduction in bone mass (osteomalacia), reduce blood cholesterol levels, and increase HDL levels, whereas saponins are proven to be efficacious as anticancer, antimicrobial, and reduce cholesterol in serum (osteomalacia), reduce blood cholesterol levels, and increase HDL levels, whereas saponins are proven to be efficacious as anticancer, antimicrobial, and reduce cholesterol in serum (osteomalacia) (Bidura *et al.*, 2017) [8, 9]. Hestera (2008) [30] and Cervantes-Valencia (2015) [14] report that the use of phytochemical compounds from Moringa oleifera leaves and Curcumin leaves in feed can reduce the cholesterol content of chicken meat. Xanthophil compounds, such as lutein, zeaxanthin, meso-zeaxanthin, astaxanthin, and canthaxanthin are carotene oxygen derivatives (Von-Lintig, 2013) [64]. Teteh *et al.* (2013) [62] that increased levels of saponins as antinutrients can reduce digestion and absorption of nutrients, especially lipids.

Yolk cholesterol levels were significantly lower in chickens given 2-4% MLF extract in drinking water compared to controls. Manipulation of the digestive tract microflora with probiotics, can play an important role in changing the metabolism of chicken lipids, because various studies have shown that probiotics can reduce cholesterol levels in egg yolks and serum (Ezema and Eze, 2015; Sun *et al.*, 2015) [21]. *Saccharomyces spp.* has a high hydrolytic activity of bile salts, which is responsible for the de-conjugation of bile salts (Bidura and Siti 2017) [8, 9] and has the ability to reduce serum cholesterol levels (Kusumawati *et al.*, 2003) [35]. Fermented feed products significantly reduce fat and cholesterol content in breast meat, but do not significantly affect water content, protein, and ash (Hasanuddin *et al*., 2017) [28]. Ezema and Eze (2015) [21] reported that supplementation of 1.0 g/kg of probiotic feed was recommended to effectively reduce serum and egg cholesterol levels and optimal chicken egg performance.

Fermentation of feed ingredients apparently increases the nutritional value of feed, as reported by Santoso *et al.* (2016) [53]; and Sugiharto *et al.* (2017) [58] can significantly increase the nutritional content of feed, and can improve the performance of poultry compared to non-fermentation. Beta-carotene can be improved in fermentation with mushrooms (*Trichoderma harzianum*), because this fungus is carotenogenic (Producing beta-carotene) (Ma *et al.,* 2000; Hirschberg, 2001; Hsieh and Yang, 2003) [39, 31, 32] The ability of beta-carotene to reduce cholesterol is related to the hydroxyl methyl glutaril-CoA enzyme (Wang, and Gu, 2010) [65].

Some research data shows that probiotics added to animal feed or drinking water can prevent infection and colonization of pathogens in the digestive tract of livestock (Bidura *et al.*, 2019) [11]. Probiotics can maintain microbial balance in the digestive tract, namely through the mechanism of competitive exclution, namely competition between pathogenic bacteria and probiotic microorganisms, so that pathogenic bacteria cannot live in the digestive tract and will come out with excreta (Zurmiati *et al.,* 2014). In addition, fermentation products affect the ecology of bacteria in the digestive tract and reduce the level of Enterobacteriaceae in various parts of the digestive tract of broiler chickens (Heres *et al.*, 2003) [32]. The same thing was reported by Hasan *et al*. (2016) [27] that the use of wet fermented feed products with probiotic microbes can cause an increase in chicken production performance.

Conclusion

We conclude that supplementation of *Moringa oleifera* leaf water extract fermented by *Saccharomyces sp* at level 2-4 cc/100 cc drinking water in laying hens Lohmann Brown can improve egg production and reduce yolk cholesterol content in laying hens

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Conflict of interest declaration

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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