Research Article

Bacteriocin Activity of Yogurt Probiotics on Increasing Production of Laying Hens

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Abstract | Bacteriocin compounds from probiotic yoghurt can make an important contribution to maintaining and improving animal health, so it is hoped that by providing probiotic yoghurt, egg production will increase. This study aims to determine the bacteriocin activity of probiotic yogurt and the effect of giving probiotic yogurt on the feed conversion ratio and total production of laying hens. This research used a Completely Randomized Design (CRD) with three treatments and twelve replications. Treatment consisted of T0: basal feed (BS), T1: BS + 4% WSPE B1 yogurt (*Bifidobacterium spp. and L. acidophilus*), and T2: BS + 4% WSPE B2 yogurt (*L. bulgaricus, S. thermophilus, L. acidophilus, and Bifidobacterium spp.*). All data from the results of sample analysis have been analyzed using analysis of variance (ANOVA), to determine the effect, and Duncan's multiple range test has been used to determine differences between treatments. In the purification of WSPE yogurt, both B1 and B2 each produced five peaks, namely peaks 1 and 2 (class II bacteriocins), peaks 3 and 4 (class I bacteriocins), and peak 5 (free amino acids). The results of the bacteriocin activity test showed that B1 had higher bacteriocin activity than B2. The result of the current reaserch show that giving WSPE yogurt, both B1 and B2, has not been able to provide a significant difference in reducing feed conversion and increasing egg production in laying hens.

Keywords | Probiotics, Bacteriocin, Layer, Hen day production, Feed conversion ratio, Egg weight

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INTRODUCTION

Laying chickens are a type of poultry cultivated to produce eggs as a source of animal protein. One of the challenges in cultivating laying hens is the risk of infection by pathogenic bacteria. Pathogenic bacteria can influence the body's immune response by stimulating the production of proinflammatory cytokines such as IL-1β, which can then trigger an inflammatory response (Duque and Descoteaux, 2014). Elevated levels of proinflammatory cytokines can cause chronic inflammation that disrupts reproductive hormonal balance. Previous studies have shown that a cytokine increase can be triggered by pathogenic bacteria in the intestine (Situmeang *et al.*, 2023). This can inhibit egg production by disrupting the function of ovarian cells and reducing the sensitivity of gonadotropins, namely FSH (follicle-growing hormone) and LH (luteinizing hormone), to the reproductive glands (Duffy *et al.*, 2019). In response to inflammation, the body can also produce glucocorticoids that suppress lymphocyte

production, stimulating increased inflammation. Increased levels of glucocorticoids can cause stress and inhibit reproductive function (Whirledge and Cidlowski, 2010). Glucocorticoids, such as cortisol, also inhibit the release of gonadotropin hormone (GnRH), which plays a role in stimulating the synthesis and release of LH and FSH from the pituitary gland. Glucocorticoids can also directly affect the ovarian glands, reducing FSH and LH production or reducing the sensitivity of ovarian cells to these hormones (Bhaumik *et al*., 2023). FSH and LH are gonadotropin hormones that are important for the reproductive process in chickens. FSH stimulates ovarian follicle growth and estrogen production, while LH stimulates ovulation and corpus luteum formation (Bosch *et al*., 2021). Thus, excessive production of proinflammatory cytokines and glucocorticoids can disrupt egg production in laying hens by disrupting the reproductive hormonal balance and function of ovarian cells, including the regulation of sensitivity to FSH and LH.

One way to prevent infection by pathogenic bacteria is by using probiotic yogurt in the ration. Probiotics have the potential to improve the structure of intestinal villi to increase absorption capacity, as well as speed up metabolism and the formation of hormones that contribute to increased egg production. Yogurt, as a source of probiotics, contains lactic acid, bacteriocins, and antioxidants, which can improve digestion and chicken egg production by inhibiting the growth of pathogenic bacteria. Water-soluble peptide Extract (WSPE) in probiotic yogurt is a water-soluble peptide extract obtained from yogurt. WSPE has antibacterial activity, such as bacteriocin, which is beneficial for health (Taha *et al*., 2017). Bacteriocins are peptides or protein compounds released extracellularly by lactic acid bacteria and have bacteriostatic and bactericidal effects on pathogenic bacteria (Urnemi *et al*., 2011). This bacteriocin compound can control intestinal pathogens through competition for nutrients and adhesion sites (Adriani *et al*., 2023). Bacteriocins have a precise mechanism for targeting pathogenic bacteria because they only affect certain types of bacteria with receptors that match the bacteriocin (Pérez-Ramos *et al*., 2021). This allows bacteriocins to destroy pathogenic bacterial cells more selectively while leaving valuable bacteria for the livestock's body. Generally, bacteriocins interact with special receptors on the surface of pathogenic bacteria. After binding to this receptor, bacteriocins will disrupt the function of bacterial cells in various ways, such as damaging the cell membrane or inhibiting the synthesis of proteins and nucleic acids (Mastuti, 2022). This will cause pathogenic bacteria to lyse.

The FSH and LH hormones will work more optimally by reducing the number of pathogenic bacteria. Giving probiotics will help the protein absorption process to be better; this condition will cause the secretion of gonadotropin hormones, especially FSH and LH, to be optimal, and the process of follicle formation and ovulation will run well so that production will increase (Kumar and Sait, 2011). With an optimal digestive system and increased egg productivity, the need for feed consumption will be more efficient so that ration conversion will decrease.

Previous research shows that probiotics increase feed intake, hen day product, and egg weight (Getachew *et al*., 2016). However, other studies show that giving powdered probiotic yogurt to laying hens in the peak production phase has no significant effect on Feed Conversion Ratio (FCR), Hen Day Product (HDP), and Egg weight (Rosiyanti, 2023). The differences in the impact of probiotics based on the results of previous studies show that the potential for studying probiotics, especially their chemical compounds, method of administration, and biological status of livestock objects, as well as the level of administration, are study subjects that are still wide open for research.

This research aims to determine the bacteriocin activity of two types of consortia, namely consortium B1 (*L. bulgaricus, L acidophilus, S. thermophilus, Bifidobacterium bifidum*) and consortium B2 (*L. acidophilus* and *Bifidobacterium* spp.) and also their effect on ration conversion, daily production and egg weight of laying hens. We hypothesize that the use of probiotic WSPE yogurt in feed can increase daily production and egg weight and can reduce feed conversion values.

MATERIALS AND METHODS

Probiotic yogurt preparation

In this research, two types of consortia were used, namely B1 and B2. The bacterial culture used for consortium B1 was *Lactobacillus acidophilus* and *Bifidobacterium* spp. and consortium B2, namely *Streptococcus thermophilus, Lactobacillus bulgaricus, Lactobacillus acidophilus*, and *Bifidobacterium bifidum* as much as 7.5% (v/v) was inoculated into 250 mL of De Man Rogosa and Sharpe media. (MRS) and then incubated at 37°C for 24 hours. Fresh milk from KSBU Lembang is pasteurized by heating at a temperature of 70-80 °C. Milk that has been heated is then cooled to a temperature of 45 °C, and the milk fermentation process was carried out to which consortium bacteria had been added for 14 hours.

Making water-soluble peptide extract (WSPE) yogurt

To obtain WSPE, the yogurt sample was centrifuged at a speed of 10,000 rpm with a temperature of 4°C for 10 minutes. The supernatant obtained was filtered using a 0.45 µm membrane filter (filter paper). Then the WSPE obtained in the form of a filtrate was dried and concentrated

using a food dehydrator at a temperature of 37 °C for two days. The dried WSPE samples were stored in a desiccator for further analysis.

Purification of water-soluble peptide extract (WSPE) yogurt

Purification was carried out using the gel filtration chromatography method. This purification aims to determine the peptide profile of WSPE yogurt (Nelson *et al*., 2008). The Sephadex G-25 matrix was inserted into a gel filtration column (60x2cm) and the water rate was 1 mL/minute. The sample in the form of WSPE yogurt with a concentration of 100,000 ppm was added to 2% of the matrix volume, then eluted using acetate buffer pH 4.5, then fractionated, and each fraction volume was 4 mL. The fraction with larger molecules will come out earlier because it can only flow around the gel, while the smaller fraction will flow more slowly because it can penetrate the gel pores. The absorbance of the fraction obtained was measured using a UV spectrophotometer at a wavelength of 280 nm and a biuret test was carried out at a wave of 535 nm using a UV-Vis spectrophotometer. The spectrum results are determined by looking at the peaks which indicate the presence of peptides with a molecular weight of 1-5 KDa in the fraction.

Determination of antibacterial activity

The bacterial test suspension resulting from inoculation on 100 µL of NB media was added to 18 mL of sterile NB media in an Erlenmeyer. Place the negative control (aquades), positive control (tetracycline), and WSPE samples with varying concentrations of 10,000 ppm and 1000 ppm, as well as the fractionated peaks (P1, P2, P3, P4, and P5) into an Erlenmeyer containing sterile NB media to which the test bacterial suspension is added. as many as 2 mL samples. The absorbance was measured with a spectrophotometer at λ= 600 nm every 2 hours for each sample and control tested.

Experimental designs

The animals used in this research were 31-weeks old ISA Brown strain laying hens, which were reared for six weeks. This research used a Completely Randomized Design (CRD) consisting of 3 treatments, where this treatment would be repeated 12 times with each cage containing one chicken for a total of 36 chickens. Each cage is labelled with a treatment and repetition number to facilitate observation and data collection. The feed used in this research is commercial feed. Feeding was done twice in the morning (7 am) and afternoon (4 pm) at 120 grams/head/day. Meanwhile, drinking water is provided as an *ad libitum*. Every day, eating and drinking places are cleaned to prevent disease. The list of treatments can be found in Table 1.

Table 1: List of treatments.

Category	Treatment
T0	BS (without treatment)
T1	BS + 4% B1 WSPE yogurt
T2	BS + 4% B2 WSPE yogurt

Data collection

Eggs are harvested daily, and the remaining chicken feed and egg weight are weighed daily. Hen Day Production (HDP) measures the average number of eggs produced per day by a chicken. Ration conversion calculates the feed required to produce one kilogram of eggs. At the end of the research, all data is processed using the following formula:

$$FCR = \frac{Feed\ intake\ (g)}{egg\ mass\ (g)}$$

$$HDP\ (\%) = \frac{Egg\ production\ a\ day}{number\ of\ hens\ at\ that\ time} \times 100$$

Statistical analysis

The data obtained were analysed using analysis of variance (ANOVA). Duncan's multiple range test is carried out if there is a difference in effect between treatments statistical analysis using SPSS Statistics 22.0 for Windows.

RESULTS AND DISCUSSION

Purification of WSPE yogurt

This purification aims to obtain peptide compounds contained in yogurt. Fractions were analysed at a wavelength of 280 nm and biuret test at a wavelength of 535 nm. The results of purifying WSPE yogurt using the gel filtration column chromatography method are shown in Figures 1 and 2.

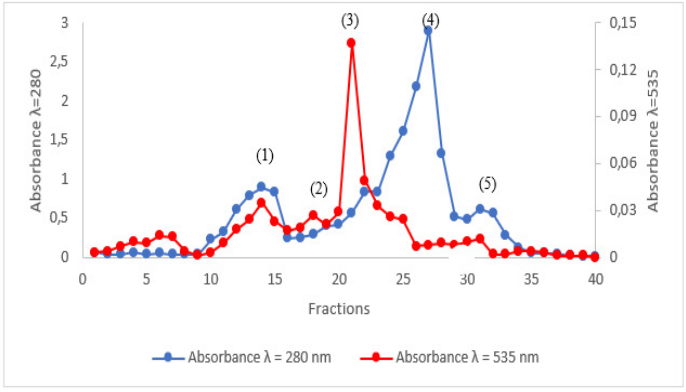

Figure 1: Protein and peptide profiles resulting from fractionation of WSPE yogurt B1 using column chromatography Sephadex G-25.

Purification using gel filtration chromatography aims to separate proteins or peptides based on their molecular weight. Absorbance measurement at a wavelength of 280 nm and the Biuret test at a wavelength of 535 nm are

two methods commonly used to estimate concentration in solutions. At a wavelength of 280 nm, tyrosine and tryptophan in the protein structure will absorb strong UV light (Biter *et al*., 2019). Meanwhile, in the biuret test, there is an interaction of peptide bonds from the WSPE yogurt purification fraction with copper ions (Cu2+) in an alkaline environment so that a purple color is formed, the intensity of the resulting purple color is correlated with the total protein concentration in the solution (Bianchi-Bosisio, 2015). These two methods are carried out together to obtain complete and accurate information about the protein concentration in solution.

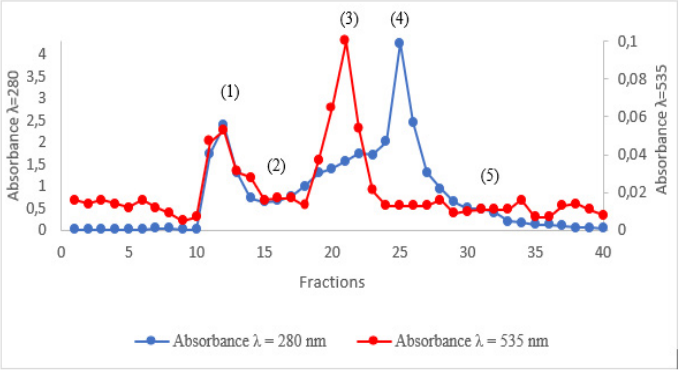

Figure 2: Protein and peptide profiles resulting from fractionation of WSPE yogurt B2 using column chromatography Sephadex G-25.

Based on the results of gel-filtration chromatography analysis at 280 nm and 535 nm waves, five peaks were found in each WSPE yogurt B1 and B2. The number of peaks in this study is higher than the research results from Waluyo *et al*. (2007), which obtained only two peaks using the Sephadex G-100 matrix. Sephadex G-100 has a higher cutoff, namely <30 kDa. In peak one, B1 (fraction 11-15) and B2 (fraction 11-15), followed by the second peak, B1 (fraction 16-19) and B2 (fraction 16-18), which are residues from the first peak. These two peaks come out first from the column so that it can be predicted that peaks one and two are protein samples with a molecular weight (>5 kDa) because they can pass directly through the matrix, according to Walls and Walker (2017), which states that Shepadex G-25 has a range of 1-5 kDa fractionation so that the buffer will directly elute proteins with large molecules (>5 kDa). The third peak of samples B1 (fraction 20-23) and B2 (fraction 19-23) had the highest absorption in the Biuret test compared to the other peaks, indicating that the peptide bonds in these peaks had the highest intensity. The four peaks B1 (fraction 24-29) and B2 (fraction 24-29) are peptides because they do not react with the Biuret test reagent but have high aromatic ring bonds, so the absorbance value produced at 280 nm shows a high value. Meanwhile, peak five B1 (30-34) and B2 (30-33) are peptides or free amino acids with lower aromatic ring bonds than peak four.

The purification results show that consortia B1 and B2 have similar peaks. Analysis using two waves, namely 280 nm and 535 nm, shows significant peaks at each wave. From the research results above, it can be seen that each peak has the potential to have bacteriocin activity. In this study, peaks 3 and 4 have the potential to have high bacteriocin activity compared to other peaks. This can be seen from peak 3, which has the highest absorption in the Biuret test, identifying high levels of peptide bonds, thus increasing the bacteriocin concentration. Meanwhile, peak 4 has high absorption at the 280 nm wave. Testing at a wavelength of 280 nm shows the presence of aromatic group amino acids, one of which is tryptophan which can produce bacteriocin activity (Reinmuth-Selzle *et al*., 2022). Purification of peptides by gel chromatography is an important step in classifying bacteriocins based on their peptide molecular weight.

Bacteriocin activity of WSPE yogurt

Tests for bacteriocin activity on WSPE and peak fractionation results of consortia B1 and B2 were carried out on Gram-negative *E. coli* bacteria and Gram-positive *S. aureus*. The positive control was tetracycline with a final concentration of 100 ppm, while the negative control used distilled water. The bacteriocin activity of WSPE and peak fractionation results from measuring optical density values are shown in Table 2.

Table 2: The bacteriocin activity of WSPE and peak fractionation.

Sample	μ max *E. coli*		μ max *S. aureus*	
	B1	B2	B1	B2
	OD/hour			
Control (+)	-0.013	-0.015	-0.010	-0.009
Control (-)	0.139	0.140	0.138	0.143
WSPE 10^4 ppm	0.023	0.045	0.028	0.040
WSPE 10^3 ppm	0.078	0.090	0.084	0.119
Peak 1	0.137	0.136	0.137	0.139
Peak 2	0.135	0.137	0.137	0.140
Peak 3	0.108	0.126	0.112	0.131
Peak 4	0.093	0.122	0.107	0.128
Peak 5	0.135	0.138	0.137	0.141

Note: C (-): negative control; C(+): Tetracycline positive control; WSPE: Water Soluble Peptides Extract; P1, P2, P3, P4, P5: Peak purification results (Peak one to five).

Bacteriocin activity testing was done using the OD (Optical Density) method. Bacteriocin activity testing was done using the OD (Optical Density) method. OD measurements are used to monitor the growth of pathogenic bacteria over a certain time. The working mechanism of OD is to monitor changes in the turbidity or turbidity of the solution. Sample turbidity will usually

increase as the number of pathogenic bacteria continues to multiply. The higher the turbidity of the sample, the higher the absorbance or OD value measured. Changes in OD values can indicate the effectiveness of bacteriocins in inhibiting the growth of pathogenic bacteria.

It can be seen from both WSPE B1 and B2 that WSPE 10^4 ppm has a higher barrier than WSPE 10^3 ppm. This proves that the higher the concentration, the higher the barrier produced by bacteriocins against pathogenic bacteria. The smaller the OD/hour value produced, the more effective the bacteriocin contained in the sample. From the peak fraction results, peaks 1 and 2 have relatively more minor barriers than peaks 3 and 4. Peaks 1 and 2 have a molecular weight (>5 kDa), so they are likely to be included in class II bacteriocins. Class II bacteriocins, with their amphiphilic helical structure, will make it easier for them to enter the target cell membrane so that the cell experiences damage and even death (Kaur and Kaur, 2015; Adriani *et al*., 2021). Meanwhile, peaks 3 and 4 with a molecular weight (<5 kDa) belong to class I bacteriocins. Class I bacteriocins have the advantage of being able to kill pathogenic bacteria using two different methods. The first way bacteriocins work is by binding to lipid-II, which plays a role in transporting peptidoglycan subunits, disrupting the cell wall synthesis process and causing cell death. The second way bacteriocins will use lipid-II to enter the membrane and form a pore, which also causes cell lysis quickly (Negash and Tsehai, 2020).

In this study, sample B1 (*L. acidophilus* and *Bifidobacterium* spp.) had higher bacteriocin activity than B2 (*L. bulgaricus, S. thermophilus, L. acidophilus*, and *Bifidobacterium bifidum*) in inhibiting the growth of pathogenic bacteria, both on gram-positive bacteria and gram-negative bacteria. This is in line with the statement of Adrini *et al*. (2008) that *L. bulgaricus* and *S. thermophilus* are not among the reliable probiotic groups in producing antimicrobials compared to *Bifidobacterium* and *L. acidophilus*. B1 bacteria are better used as probiotics for chickens because they resist acidic conditions. In laying hens, the greatest speed of digestion is in the anterior part of the small intestine. However, before reaching the small intestine, something digested by the chicken must pass through the verticulus, which has acidic conditions with a pH ranging from 3-4 so that the microbes used as probiotics must be resistant to acidic conditions and bile salts (Manin, 2012). *Bifidobacterium* and *L. Acidophilus* tolerate acids and bile salts, so they can survive in the digestive tract and maintain their probiotic activity. *L. bulgaricus* and *S. thermophilus*, on the other hand, do not belong to the native gut flora, so they are not bile acid tolerant and cannot survive passage through the gut (Gao *et al*., 2022).

Effect of WSPE yogurt on feed conversion ratio, HDP, and egg mass

Ration conversion is an indicator of efficiency of use and quality ratio. A smaller value indicates feed efficiency in egg production, while a larger value indicates waste because feed is not optimal for egg production. Effect of WSPE yoghurt on feed conversion, hen day production, and egg mass can be seen in Table 3.

Table 3: Effect of WSPE yogurt on feed conversion, hen day production, and egg mass.

Experimental	Group		
	T0	T1	T2
Feed conversion ratio	2.02^{a}+0.04	2.13^{b}+0.07	2.00^{b}±0.06
Hen day production	93.65±2.88	95.63±2.76	92.27±3.25
Egg weight	57.01±1.65	54.82±1.82	57.61±1.98

Note: Sig^{a}: 0.466; Sig^{b}:1.000

The 5% ANOVA test results showed that WSPE probiotic yogurt significantly affected feed conversion ($p<0.05$). However, they did not significantly affect hen day production and egg mass ($p>0.05$). Ration consumption variations and egg weights between individual laying hens cause differences in FCR values. Higher feed consumption and lower egg weights tend to result in higher FCR, while lower feed consumption and higher egg weights result in lower FCR (Clark *et al*., 2019). In this study, significant changes occurred in treatment T1, where there was an increase in the FCR value due to the egg weight being relatively minor compared to other treatments. However, there was a tendency for feed conversion to decrease at T2, meaning that probiotic administration in this treatment was optimal. Probiotics can produce bacteriocins, which can help maintain the balance of microbiota in the digestive tract of laying hens by inhibiting the growth of pathogenic bacteria and supporting the growth of good bacteria. In optimal microflora balance, probiotics can increase feed digestibility so that more nutrients are absorbed and used by laying hens. This makes feed use more efficient and produces eggs with greater weight so that FCR will decrease.

Several researchers have shown that the addition of probiotics in feed can reduce ration conversion and increase egg production in laying hens (Kumalasari *et al*., 2023). However, in this research, giving probiotics did not significantly affect the production of laying hens. However, there was a tendency to increase in treatment P1. The lack of effect of giving probiotics was due to the age of the livestock, which was 31 weeks old when the livestock was at peak production. At peak production, genes that regulate hormones, such as FSH and LH, become active. Optimal FSH and LH levels can stimulate rapid follicular

growth, producing optimal eggs (Rahmania *et al*., 2022; Tanuwiria *et al*., 2022; Prastiya *et al*., 2022). Therefore, giving probiotics is considered not to significantly affect the production of laying hens. These results align with the statement of Rosiyanti *et al*. (2023), who state that the genes involved in egg production will be in good condition when laying hens reach peak production, so when given probiotics, the effect will not be optimal. Even though it has no real effect on production, giving probiotics affects the health of organs such as the liver and kidneys. This can be seen from the results of joint research with different parameters, which resulted in a reduction in Serum Glutamic Oxaloacetic Transaminase (SGOT) levels from 18.86 U/L to 10.90 U/L and 8.55 U/L, Serum Glutamic Pyruvic Transaminase (SGPT) from 10.61 U/L to 9.46 U/L and 7.78 U/L and creatinine from 0.27mg/dL to 0.21mg/dL and 0.23mg/dL. This is in line with the statement of Selvamet *et al*. (2010), which states that low SGOT, SGPT, and creatinine levels indicate that liver and kidney cells are not damaged. Increased creatinine levels indicate impaired kidney function and low glomerular filtration ability (Adriani *et al*., 2017; Mushawwir *et al*., 2021).

If seen as a whole, although the provision of probiotics has not had an effect on improving the performance of laying hens, the potential of these probiotics can be superior to their bacteriocin content. As previously explained, this potentially active peptide is able to prevent inflammation and is effective, as has been reported by many previous studies. Therefore, in applying probiotics, it is necessary to give WSPE yogurt to laying hens during the final layer phase.

CONCLUSIONS AND RECOMMENDATIONS

In the purification of WSPE yogurt, both B1 (*Lactobacillus acidophilus* and *Bifidobacterium spp*.) and B2 (*L. bulgaricus, S. thermophilus, L. acidophilus*, and *Bifidobacterium bifidum*) each produced five peaks, namely peaks 1 and 2 (class II bacteriocins), peaks 3 and 4 (class I bacteriocins), and peak 5 (free amino acids). The results of the bacteriocin activity test showed that B1 had higher bacteriocin activity than B2. Giving WSPE probiotic yogurt, both B1 and B2, has not been able to provide a significant difference in reducing feed conversion and increasing egg production because the chickens used in the research were chickens at peak production. In this study, the specific peptide molecular weight of each peak of the purification product is not yet known, and the effectiveness of giving WSPE yogurt on feed conversion, hen day production, and egg weight is also not known for certain. Therefore, further research regarding specific molecular weight measurements using the SDS-PAGE method and giving WSPE yogurt to laying hens during the culling period needs to be carried out.

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NOVELTY STATEMENT

The purification and bacteriocin activity test of WSPE yogurt and supplementation of WSPE yogurt in chicken feed, as well as its effects on feed conversion and increase in egg production, have been studied, and excellent results have been demonstrated. From the purification results, we can classify bacteriocins based on the molecular weight of their peptides. From this research, we can find the characterization and activity of the bacteriocins produced by each consortium. Both consortia B1 (*Lactobacillus acidophilus* and *Bifidobacterium spp*.) and B2 (*L. bulgaricus, S. thermophilus, L. acidophilus*, and *Bifidobacterium bifidum*) have bacteriocin activity that can inhibit the growth of pathogenic bacteria. Even though the administration of probiotics has not had an effect on improving the performance of laying hens, the potential of these probiotics can be enhanced by their bacteriocin content. As previously explained, this potential active peptide is able to prevent inflammation and is effective, as has been reported by many previous studies.

AUTHOR'S CONTRIBUTION

All authors contributed equally to the writing of this manuscript.

Conflicts of interest

The authors have declared no conflict of interest.

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