Bacillus amyloliquefaciens CU33 fermented feather meal-soybean meal product improves the intestinal morphology to promote the growth performance of broilers

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ABSTRACT This study is aimed to select optimum keratin degradation ability from *Bacillus* strains for feather meal-soybean meal fermentation, and favorably water content for the strain during fermentation of feather meal-soybean meal, and finally investigate the effects of the fermented feather meal-soybean meal product (**FFSMP**) on growth performance, carcass trait, clinical blood biochemistry, and intestinal morphology of broilers. Thirty-six bacteria strains from soil, sewage pool, and feather waste were screened and selected *Bacillus subtilis* var. *natto* N21 (**N21**), *B. subtilis* CU14 (**CU14**), and *B. amyloliquefaciens* CU33 (**CU33**) with better keratinase activity and feather-degrading rate. The result of trial 1 showed that the FFSMP produced by CU33 had the optimum physiochemical characterizations, amino acid composition and feeding performance for broilers. Hence the effects of water content (45, 50, 55, and 60%) on FFMSP fermentation of CU33 were investigated in trial 2. Result showed that pH value, counts of *Bacillus*-like bacteria, γ-PGA, viscosity, surfactin yield and odor all significantly increased according to the water content ($P < 0.05$). The protease activity reached significantly highest in the 55% and 60% water content groups ($P < 0.01$). The broilers performance of 55% and 60% water content group were significantly higher than control group ($P < 0.05$) in weight gain (**WG**), feed intake (b) at 0 to 21-d-old and the WG, feed conversion ratio (**FCR**), and production efficiency factor at 0 to 35-d-old, and could reach the similar growth performance as fish meal group ($P > 0.05$). The fermentation groups significantly decreased urea nitrogen ($P < 0.05$) and increased creatinine ($P < 0.05$) in the blood. The fermentation groups also significantly decreased the crypt depth in the duodenum ($P < 0.05$) and increased villus height to crypt depth ratio of the duodenum ($P < 0.05$). In conclusion, CU33 shows the best degradation rate for feather and keratinase activity, and the FFSMP with a water content of 50% to 60% during fermentation is suggested. Diets supplemented with 5% FFSMP can promote the growth of broilers by improving the morphology of the duodenum and achieve the feeding effect of high-quality fish meal.

Key words: *Bacillus amyloliquefaciens* CU33, broiler, feather meal-soybean meal product, growth performance

INTRODUCTION

Feather waste is a by-product of the poultry industry, which contains 5% to 10% of body weight in poultry. Feathers contain about 90% of CP with primarily (around 90%) keratin (Daroit and Brandelli, 2013; Verma et al., 2017; Callegaro et al., 2019). Feathers are highly resistant to various proteolytic microorganisms, enzymes, chemical and physical damage (Nnolim et al., 2020). Therefore,

feathers are not easy to be digested and utilized by monogastric animals. Pressurized cooking is the primary processing method to produce feather meal, which can improve the degradation rate of the feather, but still has a negative impact on protein utilization for monogastric animals (Callegaro et al., 2019). High temperature in heat treatment also cause the loss or destruction of major amino acids such as methionine, lysine, and tryptophan, forming lanthionine and lysinoalanine, resulting in reduced bioavailability and potential toxicity (Fakhfakh et al., 2011; Kang et al., 2018; Hassan et al., 2020). Hence, the recommended usage of feather meals is limited to a maximum of 6% in broiler diets (Babatunde et al., 2021).

The transformation of feather meal by microorganisms can increase the digestibility of feather keratin, and

produce limiting amino acids by microorganism during degradation, so it is superior to the general hydrolysis method (Saarela et al., 2017). There are many different keratin-degrading microorganisms, and the degrading rate of bacteria is better than that of fungi (Sivakumar and Raveendran, 2015; Callegaro et al., 2019), and *Bacillus* spp. has the best potential to degrade the keratin (Bose et al., 2014; Verma et al., 2017). Solid-state fermentation (**SSF**) can decompose macromolecular substances into small molecules by microorganisms. For example, *Bacillus* spp. can reduce the content of antinutritional factors in soybean through SSF, thereby improving the nutritive value of soybean (Teng et al., 2012; Chi and Cho, 2016). Our previous study has reported that the complete broiler feed ration under the first stage 2 d aerobic fermentation with *Bacillus subtilis var. natto* N21 (N21) and second stage 3 d anaerobic fermentation with *Saccharomyces cerevisiae* Y10 or *B. coagulans* L12 had significantly improved growth performance (Chen et al., 2009; Yeh et al., 2018). Among them, the use of N21 as a single strain to produce fermented complete feed can also improve the growth performance of broilers. N21 is not a strain for keratin degradation, therefore, screening more suitable strains for feather meal fermentation is necessary.

This study is aimed to select optimum keratin degradation ability from *Bacillus* strains for feather meal-soybean meal fermentation, and favorably water content for the strain during fermentation of feather meal-soybean meal, and finally investigate the effects of the fermented feather meal-soybean meal product (**FFSMP**) on growth performance, carcass trait, clinical blood biochemistry, and intestinal morphology of broilers.

MATERIALS AND METHODS
Screening for Feather-Degrading Bacteria

In the experiment, N21 was used as a control group, which was screened from the soil, sewage pool and feather waste. The colony was incubated in TSB medium (Tryptone Soya Broth, HIMEDIA, Mumbai, MH, India), and aerobic fermentation was conducted at 55°C for one day. There were 36 strains reached a *Bacillus*-like count of 1×10^9 cfu/mL of *Bacillus*-like counts. After incubation for 2 d, only 2 strains had a higher feather hydrolysis rate than N21 (>62.3%). After identification, they were CU14 and CU33. Subsequently, the strains were measured the degradation rate of feather and keratinolytic activity.

Feathers Degradability

The feathers degradability followed the description of Huang et al. (2021) with some modifications. Add 50 mL TSB medium (Tryptone Soya Broth, HIMEDIA) and 3% feathers into a 250 mL flat-bottomed Erlenmeyer flask. After sterilization (121°C, 20 min), inoculate 5% N21, CU14 or CU33 at 10^9 cfu/mL, and incubate at 37°C, 100 rpm, for 0, 24, 48, and 72 h. The hydrolysate was passed through a filter paper (No. 1 filter paper, ADVANTEC, Tokyo, Japan) to remove unhydrolyzed feathers.

The feather degradability was calculated using the following formula (1):

Feather degradation rate(%)

$$= (A - B - C)/A \times 100 \tag{1}$$

Where A is the dry weight of the feathers before degradation, B is the dry weight of the feathers and filter after degradation, and C is the dry weight of the filter. The test was performed four replicates (n = 4).

Keratinolytic Activity Assay

The keratinolytic activity was assayed, followed by Huang et al. (2021). The reaction was incubated at 50°C for 60 min, terminated by adding 25 μL of 4M NaOH and centrifuged at 8,000 × g for 20 min; then, 100 μL of the supernatant was added to the wells of the ELISA plate sequentially, and the absorbance at 450 nm was measured. Using 200 μL of 1N NaOH solution as the blank, and the enzyme activity was calculated using the following formula (2):

Keratinolytic activity (U/mL)

$$= (OD - Blank)/(Reaction\ time \times 0.001)$$
$$\times concentrated\ fold \tag{2}$$

The test was performed 4 replicates (n = 4).

FFSMP Preparation

FFSMP Preparation followed the description of Huang et al. (2021) with some modifications. In trial 1, mixed feather meal and soybean meal at a ratio of 1:1 were supplied as fermentation substrate. The substrate was sterilized at 121°C, 1.21 kg/cm^2 for 20 min and cooled down to 45°C. Each *Bacillus* strain at 10^6 cfu/g of the substrate was premixed and inoculated with 60% water content to ferment aerobically at 37°C for 2 d. The fermented product was dried in an oven at 55°C. The water content of the FFSMP was below 12%, and 3 batches were produced for the current study. In trial 2, after the fermentation, substrate was sterilized and cooled down and the water content was adjusted to 45%, 50%, 55%, and 60%, respectively. The fermentation substrate was inoculated with CU33 (10^6 cfu/g), and aerobic fermentation was conducted at 37°C for 2 d. Other conditions were the same as in trial 1.

The Physiochemical Characterizations and Nutrient Composition of FFSMP

The pH of FFSMP was measured by a portable pH meter (digital pH meter, Goodly, Taiwan). The viscosity

and odor of FFSMP were evaluated on a 5-point scale where 1 is the best score (viscosity: 1 = not sticky; odor: 1 = most acceptable) and 5 is the worst score (viscosity: 5 = very sticky; odor: 5 = most unacceptable). The FFSMP was serially diluted in 0.85% NaCl and incubated on tryptic soy agar (TSA, HIMEDIA, Mumbai, MH, India) at 37°C for 24 h or on potato dextrose agar (PDA, HIMEDIA) at 28°C for 48 h, respectively. In colony counting for *Bacillus*-like colonies, and total yeast colonies expressed as colony-forming units per gram (cfu/g). The γ-PGA of FFSMP was measured by the method of Goto and Kunioka (1992). The proximate analysis and amino acid analyses of FFSMP were followed the description of Yeh et al. (2018) to analyze the water content, crude protein, gross energy, calcium, phosphorus, and amino acids of FFSMP. The protease activity of FFSMP refers to the methods of Secades and Guijarro (1999) and Oguntoyinbo et al. (2007). The surfactin yield of FFSMP was measured by the method of Sun et al. (2019). For analysis of physiochemical characterizations and proximate analysis of FFSMP, the test was performed with four replicates (n = 4). For analysis of amino acid of FFSMP, the test was performed with three replicates (n = 3).

Animal Management and Experimental Design

Four hundred 1-d-old Arbor Acres broiler chicks were used as experimental animals. In trial 1, one hundred sixty 1-d-old Arbor Acres broiler chicks, equal numbers of both sexes, were randomly assigned to dietary supplementation of 5% unfermented feather meal-soybean meal product (as control), 5% FFSMP produced by N21, CU14, or CU33. Each treatment had four replicates. The experimental period was 21 d. In trial 2, two hundred forty 1-d-old Arbor Acres broiler chicks, equal numbers of both sexes, were randomly assigned to dietary supplementation of 5% fish meal, 0% (as control), and 5% FFSMP produced by CU33 with 45%, 50%, or 60% water content during fermentation. Each treatment had four replicates. The experimental period was 35 d. Feed (Table 4) and water were provided ad libitum throughout the experimental period. The management of broilers mainly refer to the Manual of Arbor Acres (Aviagen, 2018). All the procedures used in this experiment were approved by the Institutional Animal Care and Use Committee of National Chiayi University (IACUC, protocol number 101031).

Feed Composition Analysis

Proximate feed analysis were followed the description of AOAC (1990) to analyze the moisture (method 930.15), crude protein (method 990.03), calcium (method 927.02), and phosphorus (935.59). The gross energy was measured with an adiabatic bomb calorimeter (model 356, Parr Instrument Company, Moline, IL). The test was performed four replicates (n = 4).

Growth Performance

In trial 1, BW and feed intake (**FI**) were recorded at week 0 and 3, In trial 2, BW, FI were recorded at week 0, 3, and 5. Feed conversion ratio (**FCR**) and production efficiency factor ((**PEF**) = (Survival rate (%) × BW (kg))/(age (d) × FCR) × 100) were calculated throughout the experiment. The test was performed 4 replicates (n = 4), using 10 broilers for each replicate.

Carcass Traits

In trial 2, 8 chicks of each group were euthanized at 35 d of age to measure the weights of the liver, proventriculus + gizzard, intestine (from the duodenum to rectum), abdominal fat (from the gizzard to celiac fat), breast (with bone and skin), and thigh (the fragment from femur to tibia, with bone and skin). The test was performed four replicates (n = 4), using 2 broilers for each replicate.

Cinical Blood Biochemistry

Blood samples were taken from the brachial-vein of chickens withdrawn from feed and water for 12 h at 35 d of age. After centrifuging (1,620 × g, 15 min), serum was stored at −40°C for further analysis. The blood biochemistry of serum, including the activities of aspartate aminotransferase, alanine aminotransferase, creatine kinase, lactate dehydrogenase, and the concentrations of glucose, total bilirubin (**T-Bili**), direct bilirubin (**D-Bili**), total protein, blood urea nitrogen (**BUN**), creatinine, uric acid (**UA**), cholesterol, triglycerid, were analyzed using an automatic blood chemical analyzer with Roche testing kits (Roche COBAS MIRA PLUS, Switzerland). The activities and concentrations of tested items were determined spectrophotometrically according to Akiba et al. (1982). The test was performed four replicates (n = 4), using 2 broilers for each replicate.

Intestinal Morphology

Two-centimeter fragments of the duodenum (posterior to gizzard), jejunum (anterior to Meckel's diverticulum), and ileum (anterior to ileo-cecal junction) of birds were collected at 35 d of age in trial 2. The intestinal fragments were prepared and examined using an optical microscope (Labophot-2, Nikon, Tokyo, Japan) according to Chiou et al. (1999). The villus height, villus perimeter, and crypt depth of the small intestine were measured using the method of Uni et al. (1995) with 10 points for each measurement. Villus height was based on the top of the crypt using the lamina propria of the villus. The villus perimeter was based on circumference and the distance between neighboring villi. Crypt depth was the shortest vertical distance from the villus contact point to the mucous membrane. The villus area was measured according to the formula of Sakamoto et al. (2000).

The test was performed 4 replicates (n = 4), using 2 broilers for each replicate.

Statistical Analysis

The continuous variables in feather degradation rate and keratinase activity, physiochemical characterizations and nutrient composition of FFSMP, amino acid composition analysis of FFSMP, growth performance, carcass traits, clinical blood biochemistry, and gut morphology were analyzed according to the following statistical model: $Y_{ij} = \mu + \tau_i + e_{ij}$, where Y_{ij} represents the measured value on the ith treatment in the jth experimental unit; μ is the overall mean; τ_i is the effect of ith treatment, and e_{ij} is the random error associated with Y_{ij}. The data were analyzed using the GLM procedure (SAS Institute, 2004), and the groups were compared using a one-way ANOVA with a Tukey post hoc test, where $P < 0.05$ indicated a statistically significant difference.

The scores in the physiological characterizations of FFSMP and the survival rate of growth performance were analyzed using the NPAR1WAY procedure (SAS Institute, 2004). The groups were compared using SAS macro implementation of a multiple comparison test according to Elliott and Hynan (2011), where $P < 0.05$ indicated a statistically significant difference.

RESULTS AND DISCUSSION

Feather Degradation and Keratinase Activity by the Feather-Degrading Bacteria

Table 1 showed the effects of *Bacillus* strains on feather hydrolysis rate and keratinase activity. The feather degradation ability of N21, CU14, and CU33 were significantly higher ($P < 0.05$) after inoculation and culture for 1, 2, and 3 d ($P < 0.05$) and reached the highest rate after 3 d of culture ($P < 0.05$). The feather degradation rate was in the order of CU33 > CU14 > N21 ($P < 0.05$). The keratinase activities reached peak

at the 24 h of culture ($P < 0.05$) and in the order of CU33 > CU14 > N21 > the control group ($P < 0.05$) during 1 to 3 d.

Many species of *Bacillus* spp. can secrete keratinase (Bose et al., 2014; Saarela et al., 2017). This experiment showed that these 3 strains, CU33, CU14, and N21, could secrete keratinase. The higher the keratinase activities have the higher feather degradation. The CU33 was significantly higher than the other groups ($P < 0.05$) with feather degradation rate of 62.3% in 24 h and reached a degradation rate of 76.3% after 3 d of culture. The result is similar to Huang et al. (2021) who used co-cultivate of 5 *Bacillus* species to decompose feathers. In this study, using a single species will be more competitive in production efficiency.

Trial 1

The Physiochemical Characterizations and Nutrient Composition of FFSMP
Table 2 showed the physiochemical characterizations of FFMSP of different strains. The pH value and the counts of *Bacillus*-like bacteria in the CU33 group were significantly higher than those in the other groups ($P < 0.05$) after 2 d of fermentation. The pH value, *Bacillus*-like bacterial counts, protease activity, and surfactin yield were also higher in CU33 than in the other 2 groups ($P < 0.05$) after drying. The γ-PGA content and viscosity were in the order of CU14 > N21 > CU33 ($P < 0.05$), whereas surfactin content was CU33 > N21 > CU14 ($P < 0.05$). Table 3 showed the amino acid composition of the fermented

Table 1. The effects of different *Bacillus* strains on feather degradation rate and keratinase activity.[1]

Day	Control[2]	Bacterial inoculation[3]			SEM
		N21	CU14	CU33	
		Feather degradation rate, %			
1	2.48[d]	36.8[b]	33.9[c]	62.3[a]	0.3
2	3.13[c]	62.3[b]	62.7[b]	74.4[a]	0.4
3	4.36[d]	63.7[c]	71.5[b]	76.3[a]	0.6
		Keratinase activity, U/mL			
1	20[d]	478[c]	521[b]	544[a]	4
2	20[d]	430[c]	470[b]	494[a]	5
3	21[d]	420[c]	438[b]	460[a]	4

[a-d]Means in the same row with different superscripts are significantly different ($P < 0.05$).
[1]Data are means of 4 batches of feather meal medium, each batch was tested in triplicate.
[2]Control: the feather medium is not inoculated with microorganisms.
[3]N21: feather medium inoculated with *Bacillus subtilis* var. *natto* N21; CU14: feather medium inoculated with *Bacillus subtilis* CU14; CU33: feather medium inoculated with *Bacillus amyloliquefaciens* CU33.

Table 2. Physiological characterizations of FFSMP of different *Bacillus* strains[1] (Trial 1).

	Control[2]	FFSMP[3]			SEM
		N21	CU14	CU33	
		pH value			
Initial	-	6.32	6.28	6.34	0.01
Fermentation	-	8.15[b]	8.17[b]	8.36[a]	0.01
Dry	-	6.42[b]	6.23[c]	6.63[a]	0.01
		Bacillus like, log cfu/g			
Initial	-	6.72	6.72	6.72	
Fermentation	-	8.29[b]	8.33[b]	8.65[a]	0.01
Dry	-	7.52[b]	7.39[b]	8.04[a]	0.05
		Nutrient composition of dry product			
Water content, %	10.6	10.7	10.5	10.6	0.04
Crude protein, %	63.2	63.8	63.7	63.9	0.24
Gross energy, kcal/kg	3072	3069	3038	3066	18
Ca, %	0.23	0.23	0.25	0.24	0.01
TP, %	0.53	0.54	0.55	0.57	0.01
		Physiological characteristics of dry product			
γ-PGA, %	-	2.71[b]	3.51[a]	1.98[c]	0.02
Viscosity, score	-	3.52[ab]	4.02[a]	2.12[b]	-
Odor, score	-	3.31	3.53	3.44	-
Protease activity, u/g	-	350[b]	337[b]	480[a]	5.93
Surfactin, mg/g	-	1.01[b]	0.15[c]	1.53[a]	0.07

[a-c]Means in the same row with different superscripts are significantly different ($P < 0.05$).
[1]Data are means of 4 batches of FFSMP, each batch was tested in triplicate.
[2]Control: Unfermented feather meal-soybean meal product.
[3]N21: Fermented feather meal-soybean meal product (FFSMP) inoculated with *Bacillus subtilis* var. *natto* N21; CU14: FFSMP inoculated with *Bacillus subtilis* CU14; CU33: FFSMP inoculated with *Bacillus amyloliquefaciens* CU33.

Table 3. Amino acid composition analysis of the FFSMP of different *Bacillus* strains[1] (Trial 1).

| | Control[2] | FFSMP[3] | | | SEM |
		N21	CU14	CU33	
Total amino acid, %	63.8[b]	64.9[a]	64.0[ab]	64.4[a]	0.2
Total essential amino acid, %	27.1[b]	27.1[b]	26.9[b]	27.5[a]	0.1
Arginine	4.35	4.26	4.27	4.32	0.02
Histidine	0.82[b]	0.82[b]	0.84[ab]	0.86[a]	0.01
Isoleucine	3.09	3.10	2.97	3.15	0.05
Leucine	5.75	5.73	5.73	5.81	0.04
Lysine	2.18[b]	2.18[b]	2.14[b]	2.36[a]	0.01
Methionine	0.62	0.66	0.67	0.68	0.02
Phenylalanine	3.13	3.18	3.11	3.18	0.03
Threonine	2.84	2.84	2.84	2.75	0.03
Valine	4.32	4.35	4.37	4.44	0.04
Total nonessential amino acid, %	36.7[b]	37.8[a]	37.1[b]	36.8[b]	0.1
Cysteine	1.86	1.94	1.92	1.90	0.03
Aspartic acid	5.41	5.48	5.44	5.51	0.04
Serine	6.17	6.15	6.13	6.21	0.03
Glutamic acid	8.91	9.01	9.07	8.96	0.06
Proline	4.92[b]	5.58[a]	4.95[b]	4.77[c]	0.01
Alanine	4.50	4.59	4.57	4.62	0.04
Glycine	3.07[b]	3.11[a]	3.07[b]	2.89[b]	0.04
Tyrosine	1.91	1.91	1.93	1.98	0.04

[a,b]Means in the same row with different superscripts are significantly different ($P < 0.05$).

[1]Data are means of 3 batches of FFSMP, each batch was tested in triplicate.

[2]Control: Unfermented feather meal-soybean meal product.

[3]N21: Fermented feather meal-soybean meal product (FFSMP) inoculated with *Bacillus subtilis* var. *natto* N21; CU14: FFSMP inoculated with *Bacillus subtilis* CU14; CU33: FFSMP inoculated with *Bacillus amyloliquefaciens* CU33.

feather meal-soybean meal product of the *Bacillus* strains. The contents of total amino acids in CU33 and N21 were significantly higher than those in the control group ($P < 0.05$), the total essential amino acids in CU33 were significantly higher than those in each group ($P < 0.05$), and the contents of histidine and lysine in the CU33 group was significantly highest ($P < 0.05$). However, the proline content of CU33 group was lower than each group ($P < 0.05$).

The pH value will increase after solid-state fermentation due to the production of NH3. The pH value in CU33 group was higher than the other groups, reflected a higher ability to decompose protein in CU33 through protease and feather decomposition ability. Since *Bacillus* spp. can form heat-resistant spores (Elshaghabee et al., 2017), which showing the ability of heat resistance when dried at 55°C and still maintain 7.52 to 8.04 log cfu/g in this study. Using 9 strains of *Bacillus amyloliquefaciens*, Berendsen et al. (2016) conducted a heat resistance study and showed that *B. amyloliquefaciens* still had growth performance at 57.7°C, which was similar to the result of maintaining a high viable count of CU33 after dried in this study.

γ-PGA is a polymer formed by the dehydration condensation of α-amino and α-carboxyl groups of 2 glutamic acid molecules of *Bacillus* strains, promoting calcium absorption, cellular immunity, and production of cytokines (Hyatt et al., 2010; Jelacic et al., 2020). *B. subtilis natto, B. subtilis* sp., and *B. amyloliquefaciens*

can all produce γ-PGA (Chettri et al., 2016; Luo et al., 2016), and the higher γ-PGA content, the higher viscosity of the fermented product (Cai et al., 2017; Li et al., 2021). In this experiment, both N21 and CU14 are *B. subtilis*, which contain a significantly higher γ-PGA than that of the CU33. High viscosity of the fermented feed required extensive drying time and greatly reduce the uniformity of the water content after drying especially in the lower bulkiness. Although the significantly lower γ-PGA content in CU33, but lower viscosity was beneficial to product drying and subsequent processing and utilization of the feed. The odor of fermentation product is due to the protein content of fermented substrates which were degraded by microorganisms and generate ammonia, H_2S, and other amines. The amines such as lysine and tryptophan of fermented substrate were also converted to the putrescine and indole by microorganisms fermentation (Bastos et al., 2020). The contents of lipids and carbohydrates also cause odor in fermentation (Alam et al., 2021).

Bacillus subtilis of the genus *Bacillus* has excellent alkaline protease production capacity (Sharma et al., 2017). In this experiment, FFSMP inoculated by different strains under solid-state fermentation showed the significantly highest protease activity in CU33 means that CU33 can decompose and utilize both feather meal and soybean meal. With lower protease activity, CU14 and N21 decomposed and utilized feather meal not as efficient as CU33 (Table 1). Surfactin is a secondary metabolite produced by *Bacillus* strains which increase depending on a cell density and can lead to membrane rupture and cell lysis, as well as destroy the surface properties that affect microbial adhesion (Banat et al., 2014). Therefore, surfactin has antibacterial and antibiofilm properties (Zanotto et al., 2019). In this study, CU33 had higher bacterial counts ($P < 0.05$) in the fermentation process than the other strains, thus CU33 had better surfactin production ($P < 0.05$).

During fermentation, microorganisms can convert compounds into smaller molecules, such as nitrogen-containing compounds, amino acids, peptides, or fatty acids (Sharma et al., 2020). Saarela et al. (2017) pointed out that the amino acids of these feather-decomposing hydrolysates tend to becoming part of bacterial protein especially lysine content that increased after fermentation. In this experiment, the total amino acid content of N21 and CU33 after fermentation was significantly higher than that of the control group ($P < 0.05$), and CU14 was between the 2, indicating that fermentation would change the composition of amino acids. These high total amino acids, lysine, and histidine of CU33 fermentation products may have potential used as raw materials or additives for the essential amino acids supplementation in livestock industries.

Growth Performance Table 5 showed the effects of different strains of FFMSP on the growth performance of broilers. Broilers in fermentation groups all showed significantly higher in the body weight gain and PEF than those of the control group ($P < 0.05$), However, the CU33 group was significantly better than the CU14

Table 4. Composition of experimental diets (as-fed basis).

Ingredient, %	0 to 21 d			21 to 35 d		
	Fish meal	Control	FFSMP	Fish meal	Control	FFSMP
Corn meal	48.47	48.08	48.08	58.20	57.68	57.68
Soybean meal, 44%	21.00	20.00	20.00	9.80	9.00	9.00
Full fat soybean meal, 35%	20.00	20.00	20.00	23.00	23.00	23.00
Fish meal (Peru), 65%	5.00	-	-	5.00	-	-
UFSMP[1], 63.8%	-	5.00	-	-	5.00	-
FFSMP[2], 63.8%	-	-	5.00	-	-	5.00
Ground limestone	1.20	1.60	1.60	0.80	1.40	1.40
Dicalcium phosphate	1.10	1.60	1.60	1.00	1.30	1.30
Soybean oil	2.50	2.50	2.50	1.50	1.50	1.50
Salt	0.40	0.40	0.40	0.40	0.40	0.40
Choline chloride 50%	-	0.02	0.02	-	0.02	0.02
L-Lys·HCl (78%)	-	0.40	0.40	-	0.30	0.30
DL-Met	0.13	0.20	0.20	0.10	0.20	0.20
Vitamin premix[3]	0.10	0.10	0.10	0.10	0.10	0.10
Mineral premix[4]	0.10	0.10	0.10	0.10	0.10	0.10
Total	100.0	100.0	100.0	100.0	100.0	100.0
		Calculated nutrient composition				
ME, kcal/kg		3,130			3,233	
CP, %		23.15			20.18	
Ca, %		0.97			0.83	
AP, %		0.49			0.42	
Met, %		0.56			0.50	
Lys, %		1.46			1.24	
		Analyzed values				
DM, %	92.3	92.4	92.3	91.9	91.8	91.7
GE, kcal/kg	4,403	4,400	4,396	4,531	4,535	4,531
CP, %	23.4	23.5	23.5	20.4	20.6	20.5
Ca, %	1.00	1.02	1.00	0.86	0.87	0.85
TP, %	0.67	0.68	0.67	0.63	0.65	0.63

[1]USFMP: Unfermented feather meal-soybean meal product.

[2]FFSMP: Fermented feather meal-soybean meal product is made with 45, 50, 55, and 60% water content, respectively.

[3]Vitamin premix supplied per kilogram of diet: Vitamin A, 9,000 IU; Vitamin D_3, 3,000 ICU; Vitamin E, 30 IU; Vitamin K_3, 2.2 mg; Vitamin B_1, 2.2 mg; Vitamin B_2, 8.0 mg; Vitamin B_6, 4.4 mg; Ca-pantothenate, 12.0 mg; Niacin, 66 mg, Biotin, 0.2 mg; Folic acid, 1.0 mg; Vitamin B_{12}, 0.02 mg.

[4]Mineral premix supplied per kilogram of diet: Cu ($CuSO_4 \cdot 5H_2O$, 25.45% Cu), 8 mg; Fe ($FeSO_4 \cdot 7H_2O$, 20.09% Fe), 100 mg; Mn ($MnSO_4H_2O$, 32.49% Mn), 100 mg; Zn (ZnO, 80.35%, Zn), 75 mg; Se ($NaSeO_3$, 45.56%Se), 0.30 mg.

group ($P < 0.05$), but only the CU33 group in FCR was better than the UFSMP group ($P < 0.05$).

Fermented products produced with *Bacillus* can promote growth performance in broilers (Li et al., 2014; Wu et al., 2020). Our previous studies found that either wet or dry products of N21 aerobic fermentation of complete feed can improve the growth performance of broilers (Chen et al., 2009; Yeh et al., 2018). The N21 fermented product promoting growth is again confirmed in this

experiment whereas CU14 and CU33 also had the same effect. This proves that *Bacillus* spp. used for fermentation can improve the growth performance of chickens, and CU33 had the best feather keratinase activity, degradation rate (Table 1), *Bacillus*-like bacterial counts, protease activity, surfactin yield of the fermented product (Table 2), and the largest amount of total essential amino acid, lysine, and histidine content (Table 3). We concluded that CU33 was the best for overall growth performance. Therefore, CU33 was choosen as the strain for subsequent experiments.

Trial 2

The Physiochemical Characterizations and Nutrient Composition of FFSMP

Table 6 showed the physicochemical characterizations of FFMSP fermented with different water content. During fermentation and after drying, the pH and *Bacillus*-like bacterial counts, γ-PGA, viscosity, and surfactin yield increased with the increase of fermentation water content ($P < 0.05$), while odor decreased with the decrease of water content ($P < 0.05$). The protease activity was significantly higher in the water content of 55% and 60% than that of 50% and 45% and 45% was the lowest ($P < 0.01$).

If the water content is too high, the viscosity of the fermentation product will increase causing odor and

Table 5. Effect of FFSMP of different *Bacillus* strains on growth performance of broiler during 0 to 21 days of age[1] (Trial 1).

Item	Control[2]	FFSMP[3]			SEM
		N21	CU14	CU33	
Weight gain, g/bird	736[c]	805[a]	779[b]	815[a]	8
Feed intake, g/bird	1038	1076	1059	1069	21
Feed conversion ratio, feed intake/weight gain	1.41[a]	1.34[ab]	1.36[ab]	1.31[b]	0.03
Survival rate, %	100	100	100	100	-
PEF[4]	264[c]	303[ab]	288[b]	312[a]	7

[a-c]Means in the same row with different superscripts are significantly different ($P < 0.05$).

[1]Data are means of 4 pens of broilers with 10 broilers per pen.

[2]Control: Unfermented feather meal-soybean meal product.

[3]N21: fermented feather meal-soybean meal product (FFSMP) inoculated with *Bacillus subtilis* var. *natto* N21; CU14: FFSMP inoculated with *Bacillus subtilis* CU14; CU33: FFSMP inoculated with *Bacillus amyloliquefaciens* CU33.

[4]Production efficiency factor, PEF = (Survival rate (%) × BW (kg))/ (age (d) × feed conversion ratio) × 100.

Table 6. Physiological characterizations of FFSMP fermented with different water content[1] (Trial 2).

	Water content[2], %				
	45	50	55	60	SEM
pH value					
Initial	6.13[b]	6.25[a]	6.28[a]	6.30[a]	0.03
Fermentation	8.02[d]	8.15[c]	8.32[b]	8.56[a]	0.01
Dry	6.42[c]	6.52[b]	6.53[b]	6.63[a]	0.01
Bacillus like, log cfu/g					
Initial	6.72	6.72	6.72	6.72	
Fermentation	7.84[c]	8.29[b]	8.39[b]	8.82[a]	0.05
Dry	6.20[d]	7.52[c]	7.68[b]	7.88[a]	0.02
Nutrient composition of dry product					
Water content, %	10.6	10.6	10.5	10.6	0.1
Crude protein, %	62.2	63.1	63.4	63.8	0.7
Gross energy, kcal/kg	3069	3039	3071	3066	28
Ca, %	0.21	0.23	0.25	0.23	0.02
TP, %	0.54	0.55	0.52	0.53	0.02
Physiological characteristics of dry product					
γ-PGA	1.20[d]	1.80[c]	1.90[b]	2.01[a]	0.02
Viscosity, score	1.13[b]	1.65[ab]	2.01[ab]	2.23[a]	–
Odor, score	1.30[b]	2.05[ab]	3.02[ab]	3.51[a]	–
Protease activity, u/g	270[c]	421[b]	481[a]	460[ab]	11
Surfactin, mg/g	0.45[d]	1.10[c]	1.31[b]	1.62[a]	0.03

[a-d]Means in the same row with different superscripts are significantly different ($P < 0.05$).

[1]Data are means of 4 batches of FFSMP, each batch was tested in triplicate.

[2]Fermented feather meal-soybean meal product (FFSMP) produced by CU33 with 45, 50, or 60% water content during fermentation.

drying problem. It will not be suitable for industrial production and environmental protection. The pH, bacterial count, general composition, γ-PGA, viscosity, protease activity and surfactin yield of the CU33 strain after 60% water content fermentation were similar to the results of trial 1, indicating that the fermentation process was consistent. The decreased water content will reduce the *Bacillus*-like bacterial counts, resulting in decreased pH, γ-PGA, viscosity, and surfactin yield.

Low-water content media leads to insufficient water activity, insufficient nutrient utilization, and high swelling and water tension (Jin et al., 2019). The 45% water group had insufficient bacterial count due to insufficient fermentation water, so pH, γ-PGA, surfactin, viscosity, and odor were the lowest ($P < 0.05$). Therefore, a certain amount of water is still required to make the fermentation quality reach a certain level. When fermented in the 55% water group, its protease activity was the same as that in the 60% group ($P > 0.05$), indicating that it has a particular decomposition ability.

Growth Performance Table 7 showed the effects of different water content FFMSP on the growth performance of broilers. Broilers fed diets supplemented with fermentation under 50% to 60% water content had significantly higher body weight gain and feed intake than those in the control group ($P < 0.05$) and reached the comparable level of the fish meal group ($P > 0.05$) during 0 to 21 d of age. In the 45% water content fermented group, only the body weight gain was higher than that in the control group ($P < 0.05$). During 0 to 35 d of age, the body weight gain, FCR, and PEF of diets supplemented with 50% to 60% water content fermented group were significantly higher than those in the control group ($P < 0.05$) and reached the comparable level as the fish meal group ($P > 0.05$).

The 60% water content group was significantly better than the control group in WG and FI during 0 to 21-d-old. That was the same as the result of trial 1. It was again proved that the fermented feather meal-soybean meal product of CU33 can promote the growth of broilers and achieve the comparable level of a high-quality fishmeal ($P > 0.05$). In this experiment, when the medium water content was reduced to 45%, the WG of broilers during 0 to 21-d-old was still significantly better than that of the control group ($P < 0.05$). It means that CU33 is positive on the growth performance of broilers. When its water content reached 50% or more, its effect on performance was better than the 45% group ($P < 0.05$). At the whole feeding period, there was no significant difference in growth performance between broilers fed the fermented on 50% to 60% water content group and the fish meal group ($P > 0.05$). The study proved that FFSMP fermented under fit water content could

Table 7. The effect of adding FFSMP fermented with different water content in the diet on the broiler growth performance[1] (Trial 2).

			Water content[3], %				
	Fish meal	Control[2]	45	50	55	60	SEM
0 to 21 d							
Weight gain, g/bird	769[ab]	704[c]	741[b]	790[a]	783[a]	795[a]	12
Feed intake, g/bird	1,038[ab]	979[c]	993[bc]	1,067[a]	1,060[a]	1,058[a]	18
Feed conversion ratio, feed intake/weight gain	1.34	1.39	1.34	1.35	1.35	1.33	0.02
21 to 35 d							
Weight gain, g/bird	1,156	1,130	1,134	1,142	1,185	1,180	19
Feed intake, g/bird	2,106	2,125	2,102	2,008	2,069	2,116	37
Feed conversion ratio, feed intake/weight gain	1.82	1.88	1.85	1.76	1.75	1.80	0.04
0 to 35 d							
Weight gain, g/bird	1,926[ab]	1,835[c]	1,876[bc]	1,933[a]	1,969[a]	1,975[a]	17
Feed intake, g/bird	3,144	3,104	3,095	3,076	3,129	3,174	37
Feed conversion ratio, feed intake/weight gain	1.63[ab]	1.69[a]	1.65[ab]	1.59[b]	1.59[b]	1.61[b]	0.02
Survival rate, %	100	97.5	100	100	97.5	100	–
PEF[4]	355[ab]	319[c]	343[b]	366[ab]	363[ab]	371[a]	8

[a-c]Means in the same row with different superscripts are significantly different ($P < 0.05$).

[1]Data are means of 4 pens of broilers, each pen was used 10 broilers.

[2]Control: Unfermented feather meal-soybean meal product.

[3]Fermented feather meal-soybean meal product (FFSMP) produced by CU33 with 45, 50, or 60% water content during fermentation.

[4]Production efficiency factor, PEF = (Survival rate (%) × BW (kg))/(age (d) × feed conversion ratio) × 100.

Table 8. The effect of adding FFSMP fermented with different water content in the diet on the broiler carcass traits[1] (Trial 2).

	Fish meal	Control[2]	Water content[3], %				SEM
			45	50	55	60	
Live weight, g	1,994[a]	1,897[b]	1,884[b]	2,048[a]	2,061[a]	2,047[a]	28
			Relative weight, g/100 g of body weight				
Dressing	83.2	82.1	82.3	82.4	83.0	83.0	0.5
Heart	0.57	0.56	0.57	0.56	0.54	0.55	0.03
Liver	2.28	2.47	2.32	2.42	2.44	2.35	0.07
Proventriculus + gizzard	2.82[b]	3.29[a]	2.99[b]	3.01[b]	2.83[b]	2.93[b]	0.06
Intestinal	5.54[b]	6.62[a]	6.00[ab]	6.11[ab]	6.04[ab]	6.03[ab]	0.18
Abdominal fat	1.46	1.47	1.45	1.54	1.56	1.53	0.09
Breast	18.7	18.6	18.2	18.5	18.5	19.2	0.32
Thigh	21.5	20.2	21.0	21.1	20.9	20.3	0.42

[a,b]Means in the same row with different superscripts are significantly different ($P < 0.05$).
[1]Data are means of 4 pens of broilers, each pen was used 2 broilers.
[2]Control: Unfermented feather meal-soybean meal product.
[3]Fermented feather meal-soybean meal product (FFSMP) produced by CU33 with 45, 50, or 60% water content during fermentation.

have feeding value like fish meal for broilers and have the potential to become a high-quality protein source for animal. In terms of effectively decomposing feather meal, increasing the fermentation water can obtain higher bacterial count, γ-PGA, surfactin yield, and protease activity. However, the higher the water content, the heavier the odor, and water content between 50% and 60% had no significant difference on growth performance, we suggested that 50% water content was more suitable water for the production of fermented products, environmental protection, and growth performance.

Carcass Traits Table 8 showed the effects of FFMSP with different water content on the carcass traits of broilers. The relative gizzard, and intestinal weight of the broilers in control group were significantly higher than those of the fish meal group ($P < 0.05$), and the other groups were between them ($P > 0.05$). Since fermented product is more digestible in chickens (Alshelmani et al., 2016,2017), the live weight of the fish meal group and 55% or 60% fermented supplement groups heavier than that of the control group, although feed intake had no significant difference ($P > 0.05$), Due to the less digestible ingredient of 5% unfermented feather

meal-soybean meal product in the control group caused more intensified enzyme secretion and contraction movement in digestive process, hence the relative weight of tract was higher than those of the fish meal group ($P < 0.05$), while those of the fermentation groups were between them.

Clinical Blood Biochemistry Table 9 showed the effects of different water content FFMSP on clinical blood biochemistry of broilers. The T-Bili content in the fish meal group was significantly higher than that in the other groups ($P < 0.05$). BUN and creatinine in the fermentation group were significantly lower and higher than those in the control group, respectively ($P < 0.05$).

UA, BUN, and creatinine are important indicators for evaluating animal kidney function (Abdelhalim et al., 2020). BUN is the end product of protein metabolism in the body, while creatinine is a nonprotein nitrogen product after muscle formation (Adewole et al., 2021). Since the amino acid composition and essential amino acid content of the fish meal and fermented products were significantly better than those of the control group, lower BUN content and higher creatinine content reflected a better body weight gain. The overall clinical

Table 9. The effect of adding FFSMP fermented with different water content in the diet on the broiler serum biochemical constituents[1] (Trial 2).

	Fish meal	Control[2]	Water content[3] %				SEM
			45	50	55	60	
Aspartate aminotransferase, U/L	269	254	246	256	245	229	18
Alanine aminotransferase, U/L	2.78	2.30	2.56	2.75	2.38	2.63	0.17
Creatine kinase, U/L	7,150	5,804	7,420	7,807	7,042	6,708	619
Lactate dehydrogenase, U/L	859	730	688	763	899	706	62
Glucose, mg/dL	184	173	170	193	183	200	8
Total bilirubin	0.99[a]	0.75[b]	0.83[b]	0.82[b]	0.81[b]	0.72[b]	0.05
Direct bilirubin	0.093	0.091	0.079	0.08	0.076	0.076	0.008
Total protein, g/dL	2.77	2.86	2.84	2.95	2.80	2.93	0.01
Blood urea nitrogen, mg/dL	1.64[bc]	2.05[a]	1.69[b]	1.61[bc]	1.67[b]	1.34[c]	0.10
Creatinine, mg/dL	0.39[a]	0.32[b]	0.36[a]	0.37[a]	0.36[a]	0.38[a]	0.01
Uric Acid, mg/dL	4.20	3.43	3.80	3.41	4.56	3.83	0.51
Cholesterol, mg/dL	131	127	136	134	131	133	5
Triglycerid, mg/dL	23.4	22.2	21.8	23.8	24.6	25.5	4.8

[a-c]Means in the same row with different superscripts are significantly different ($P < 0.05$).
[1]Data are means of 4 pens of broilers, each pen was used 2 broilers.
[2]Control: Unfermented feather meal-soybean meal product.
[3]Fermented feather meal-soybean meal product (FFSMP) produced by CU33 with 45, 50, or 60% water content during fermentation.

Table 10. The effect of adding FFSMP fermented with different water content in the diet on the broiler intestinal morphology[1] (Trial 2).

	Fish meal	Control[2]	Water content,[3] %				SEM
			45%	50%	55%	60%	
			Duodenum				
Villus height, μm	1,717[a]	1,448[b]	1,575[ab]	1,557[ab]	1,641[a]	1,626[ab]	43
Crypt depth, μm	177[b]	221[a]	183[b]	175[b]	164[b]	161[b]	6
Villous height: crypt depth	9.74[a]	6.59[b]	8.67[a]	9.14[a]	10.2[a]	10.2[a]	0.5
Surface area, mm^2	0.33	0.28	0.28	0.29	0.29	0.29	0.02
			Jejunum				
Villus height, μm	1,423	1,391	1,467	1,472	1,514	1,499	53
Crypt depth, μm	153	160.9	155	146	163	160	6
Villous height: crypt depth	9.39	8.74	9.21	10.1	9.34	9.37	0.35
Surface area, mm^2	0.26	0.23	0.23	0.22	0.23	0.23	0.02
			Ileum				
Villus height, μm	842	775	803	819	864	867	25
Crypt depth, μm	134	146	149	142	136	138	9
Villous height: crypt depth	6.44	5.4	5.56	6.03	6.51	6.37	0.36
Surface area, mm^2	0.15	0.13	0.14	0.15	0.16	0.16	0.008

[a,b]Means in the same row with different superscripts are significantly different ($P < 0.05$).
[1]Data are means of 4 pens of broilers, each pen was used 2 broilers.
[2]Control: Unfermented feather meal-soybean meal product.
[3]Fermented feather meal-soybean meal product (FFSMP) produced by CU33 with 45, 50, or 60% water content during fermentation.

enzymes among the groups were no significant difference ($P > 0.05$). The T-Bili in the blood includes D-Bili and indirect bilirubin, and it is usually used as an indicator of liver damage (Ruiz et al., 2021). Although the fish meal group had higher T-Bili than the other groups ($P < 0.05$), the D-Bili after liver treatment was normal. It indicated that the ability of liver to metabolize bilirubin was normal. The reason for the higher T-Bili in the fish meal group remains to be explored in subsequent studies. The fermented products produced by CU33 with 45% to 60% water content had no adverse effects on animal tissues, organs, and physiology, indicating the safety in utilization.

Gut Morphology Table 10 showed the effects of different water content FFMSP on the gut morphology of broilers. The duodenal villus height was significantly lower in the control group than that in the 55% water content group and the fish meal group ($P < 0.05$).

Deeper crypts indicate higher intestinal cell turnover (Ząbek et al., 2020) and faster tissue replacement (Berrocoso et al., 2017; Ząbek et al., 2020). Therefore, a deeper nest depth will increase nutrient require gut maintenance and reduce bird performance. In contrast, increased villus height and VH/CD ratio reflect an increase replacement and well-differentiated intestinal mucosal epithelial cells hence increase digestibility and absorptivity (Boontiam et al., 2017; Wu et al., 2020). In this experiment, the duodenal villus height, crypt depth and VH/CD ratio were the lowest in control group among all the groups reflecting the lowest 0 to 35-d-old weight gain and worst FCR performance ($P < 0.05$). It is confirmed that fermented feed can improve the duodenal tissue type, promote digestion and absorption, resulted in better chickens growth.

CONCLUSION

In conclusion, CU33 inoculated in feather meal-soybean meal fermentation shows the best degradation rate

and keratinase activity for feather. After 2 d of aerobic fermentation, the protease activity, surfactin yield, total essential amino acids, lysine, and histidine of FFSMP produced by CU33 have the best performance. FFSMP with a water content of 50% to 60% during fermentation can promote the growth of broilers by improving the morphology of the duodenum.

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The authors declare no conflicts of interest.

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