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The Effects of Two-Stage Fermented Feather Meal-Soybean Meal Product on Growth Performance, Blood Biochemistry, and Immunity of Nursery Pigs

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Abstract: The keratinase-producing microbes can readily change the feather waste into more bioavailable peptides and amino acids. This study aimed to improve two-stage fermented feather meal-soybean meal product (TSFP) quality with five selected Bacillus strains and *Saccharomyces cerevisiae* Y10, as well as investigate the effects of TSFP on growth performance, blood biochemistry, and immunity of nursery pigs. In trial 1, 100 hybrid piglets (Duroc x KHAPS) were randomly assigned to dietary supplementation of 5% fish meal or 5% TSFP prepared with 0, 32, 40, or 48 h in the first-stage fermentation. The results showed that the body weight (BW), average daily gain (ADG), and feed conversion ratio (FCR) of fermented groups were significantly better than the unfermented group ($p < 0.05$) at weeks 0 to 3 and 0 to 5. The ADG of 32-hr and 48-hr TSFP groups were better than the unfermented group ($p < 0.05$) at weeks 3 to 5. In trial 2, 80 hybrid piglets (Duroc × KHAPS) were randomly assigned into 5% fish meal or different supplementation levels of TSFP (32-hr first-stage fermented time) at 0, 2.5, or 5%. The 5% TSFP group had better BW, ADG, FCR, and PEF than the 0% group ($p < 0.05$) at weeks 0 to 5. Furthermore, the ex vivo mitogen-induced lymphoblastogenesis, the interferon-γ production, the oxidative burst activity, and the IgG production of the 5% TSFP group were higher than the fish meal group ($p < 0.05$). In conclusion, the first-stage fermentation time can be shortened from 48 h to 32 h using selected Bacillus strains in TSFP production when supplemented at 5% of the diet for nursery pigs shows the best growth performance and immunity.

Keywords: nursery pig; growth performance; immunity; blood biochemical; feather meal

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1. Introduction

High-quality fish meal is generally a primary protein source in nursery pig diets, which has the characteristics of high crude protein (CP), balanced amino acid composition, high nutrient availability, and suitable palatability [1,2]. Due to global climate change, high-quality fish meals are becoming scarce resources, which induced rising prices [3]. Therefore, finding fish meal replacement is gaining attention in animal husbandry. Feathers contain up to 85% of CP with primarily (around 90%) keratin. The disulfide bond, hydrogen bond, and hydrophobic property of keratin facilitate its low solubilities, hard to be hydrolyzed by enzymes, and the nature of the unbalanced amino acid composition [4–6]. Conventionally, the primary process of the industry to produce hydrolyzing feathers was under high temperature and high pressure, which may denature some amino acids, adversely affecting

the utilization in monogastric animals [7,8]. Hence, the recommended usage of feather meal is limited to 5% in pig diets [9].

Microbial fermentation changes macromolecules into easily digestible small-size molecules and reduces the anti-nutrient factor to improve the utilization value [10–12]. Using microorganisms to produce keratinase can effectively degrade feather keratin into bioavailable amino acids and soluble peptides to improve the nutritive value of feather meal [13,14]. Piglets have an underdeveloped digestive and immune system and face major stress during weaning. Shifting different feeding regimens and regrouping with other piglets, in addition to the depletion of passive immunity from milk, may lead to poor growth and disease infection in piglets [15]. The results of Sinn et al. [16] proved that the fermented soybean meal product could improve the growth performance and reduce post-weaning diarrhea syndrome of nursery pigs, and it also had the potential to replace fish meal in piglet diets. Additionally, most studies have demonstrated the beneficial effects of microbial fermentation products or fermented feeds to improve animal growth, feed intake, and immunity [4,17,18].

Recently, fermented soybean meal supplemented in piglets improved growth performance by reducing inflammation [4]. Our previous studies have proved that feather meal under two-stage fermented technology could be used as a protein source to substitute for fish meal in a pig diet [18]. Note that only using feather meal as a substrate is unsuitable for microbial solid-state fermentation due to the lack of carbon sources. Therefore, using soybean meal as an additional carbon source can improve solid-state fermentation quality and the amino acid balance of fermented products. Huang et al. [18] mixed feather meal and soybean meal at a ratio of 1:1 as fermented substrate under the first-stage 2-day aerobic fermentation with plant protein-degrading strain *Bacillus subtilis* var. *natto* N21 (N21) and keratinase-producing strains *B. subtilis* Da2, Da15, and *B. amyloliquefaciens* Da6, Da16, and subsequent second-stage 5-days anaerobic fermentation with *B. coagulans* L12 (L12) to produce a two-stage fermented product (TSFP). Integration of TSFP to finishing pig diets at 2.5% or 5% showed improving ADG and FCR compared with the control group and fish meal group. Moreover, the 5% TSFP had improved immune functions.

Although TSFP exhibits beneficial effects on growth performance and immunity in pigs, the two-stage fermentation product requires more time and costs, and the longer fermentation time results in the product being too viscous to dry. Moreover, the unpleasant odor further reduces the willingness to use on pig farmers. Previously, Chen et al. [19] used N21 with efficient plant protein degradability in aerobic fermentation for 48 h at the first stage and *Saccharomyces cerevisiae* Y10 (Y10) with strong acid production ability in anaerobic fermentation for 72 h at the second stage to produce a two-stage fermentation wet complete-feed. The fermentation process quickly acidified feed to a low pH of 5.5, and the feed promoted growth in broilers [19].

Therefore, it is hypothesized that using Y10 instead of L12 may reduce the time by at least 48 h at the second stage of the fermented process, but the nutritive value of modified TSFP on nursery pigs still needs to be evaluated. There are two objectives of this study. Firstly, to reduce the aerobic fermentation time and lower the viscosity and odor of TSFP at the first stage of fermentation, Y10 with strong acid production capacity to shorten the second stage of anaerobic fermentation time was used to investigate. The effects of the modified TSFP through the improved process on the growth performance and blood biochemistry of nursery pigs were studied (trial 1). Secondly, to investigate the optimal formulation of TSFP on growth performance and immunity of nursery pigs (trial 2).

2. Materials and Methods

2.1. Two-Stage Fermented Product (TSFP) Preparation

The TSFP preparation and analysis followed the description of Huang et al. [18] with some modifications. Mixed feather meal and soybean meal at a ratio of 1:1 were supplied as fermented substrate. The substrate was sterilized at 121 °C for 30 min and cooled down to 45 °C, and it was 0 h TSFP (unfermented group). *Bacillus subtilis* Da2, Da15, and *B. amy-*

loliquefaciens Da6, Da16, and *B. subtilis* var. *natto* N21, having strong protein decomposition ability were used as the first-stage fermentation strains. Each of the five Bacillus strains at 10^6 CFU/g of the substrate was premixed and inoculated with 50% w/w sterilized water to ferment aerobically at 37 °C for 32, 40, or 48 h, respectively. Subsequently, *Saccharomyces cerevisiae* Y10, with strong acid-producing ability, was inoculated in the second-stage fermentation strain at 10^6 CFU/g of the substrate. Fermentation was anaerobically at 28 °C for an additional 72 h. The fermented product was then dried using a 55 °C oven. The moisture of the final product was below 12%, and 3 batches were produced for the current study.

2.2. The Physiochemical Characterizations and Nutrient Composition of TSFP

The pH of TSFP was measured by a portable pH meter (digital pH meter, Goodly, Taiwan). The viscosity and odor of TSFP 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). A sample of TSFP 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, Mumbai, MH, India) at 28 °C for 48 h. Bacillus-like colonies and total yeast colonies were counted to express as colony-forming units per gram (CFU/g). The γ-polyglutamic acid (γ-PGA) of TSFP was measured by the method of Goto and Kunioka [20]. The proximate analysis of TSFP followed the description of AOAC [21] to analyze the moisture (method 930.15), ash (method 923.03), crude protein (method 990.03), calcium (method 927.02), and phosphorus (method 935.59). The gross energy was measured with an adiabatic bomb calorimeter (model 356, Parr Instrument Company, Moline, IL, USA). The physicochemical characterizations and nutrient composition of TSFP were analyzed with 3 replicates (n = 3).

2.3. Animal Management and Experimental Design

In trial 1, a total of 100 hybrid (Duroc × KHAPS) [22] nursery pigs (age of 35 days) with equal numbers of both barrows and females were randomly assigned into dietary supplementation of 5% fish meal or 5% TSFP prepared with 0, 32, 40, or 48 h in the first-stage fermentation. Each treatment had 5 replicates. In trial 2, a total of 80 hybrid (Duroc × KHAPS) nursery pigs (age of 35 days) with equal numbers of both barrows and females were randomly assigned into dietary supplementation of 5% fish meal or 0, 2.5, or 5% TSFP prepared with 32 h in the first-stage fermentation. Each treatment had 5 replicates. The hybrid piglets were raised on a slatted floor (the area of each pen was 2.2 m × 2.8 m) for 5 weeks during the entire experiment. Each pen (providing 1.54 m^2 of space for each piglet) had a feeder and two nipple waterers. Visit the nursery house daily at 8:00 am and 5:00 pm to ensure that the feeders and waterers are functioning properly. Floors were flush-cleaned at 11:00 am twice a week to avoid feces building up in the pens. Feed diets for pigs were formulated with reference to the nutrient requirements recommended by NRC [6]. Feed (Tables 1 and 2) and water were provided ad libitum throughout the experimental period. All the procedures used in this experiment were approved by the Institutional Animal Care and Use Committee of Kaohsiung Animal Propagation Station, Kaohsiung, Taiwan, ROC (protocol number LRIACUC-103-4).

2.4. Feed Composition Analysis

In trial 1 and trial 2, proximate feed analysis followed the description of AOAC [21] to analyze the crude protein (method 990.03), calcium (method 927.02), and phosphorus (935.59).

2.5. Growth Performance

In trial 1 and trial 2, body weight and feed intake were recorded on weeks 0, 3, and 5. Consequently, the weight gain, feed conversion ratio, and production efficiency factor were calculated and monitored throughout the experiment.

2.6. Blood Biochemistry

In trial 1, the blood samples were collected via the jugular vein with an EDTA vacutainer (BD Vacutainer, Avenue Broken Bow, NE, USA). Plasma was obtained by centrifugation at 2500× *g* for 30 min at 4 °C and stored at −20 °C for later determination of the blood biochemistry. The blood biochemistry of the plasma, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total protein (TP), blood urea nitrogen (BUN), triglycerides (TG), cholesterol (CHOL), high-density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein-cholesterol (LDL-C), were determined using a Blood Analyzer (Express Plus, Bayer, MA, USA). The plasma urea was assayed by the method of Tomas et al. [23], and plasma AST and ALT were assayed using the method of Moss and Henderson [24]. The TG, CHOL, HDL-C, and LDL-C determinations used the method of Rifai et al. [25].

2.7. Immune Characteristics

In trial 2, the assessment of lymphoblastogenesis, cytokine production, phagocytosis of granulocyte, oxidative burst measurement, blood immunoglobulin level, and lymphocyte subpopulation analysis were followed by a previous study [18]. The method details are provided in Supplementary Material S1.

Table 1. Composition of experiment diets (as-fed basis) (trial 1).

Ingredients (%)	5% Fish Meal	Fermented time, h			
		0	32	40	48
Corn meal	61.82	58.84	58.84	58.84	58.84
Soybean, full fat, cooked, 38% CP	6.0	7.2	7.2	7.2	7.2
Soybean oil	0.62	1.35	1.35	1.35	1.35
Soybean meal dehulled, solvent, 47.8% CP	21.8	21.8	21.8	21.8	21.8
Skim milk, dried	2.5	2.5	2.5	2.5	2.5
Fish meal (Peru), 65% CP	5.0	0.0	0.0	0.0	0.0
TSFP [1], 62% CP	0.0	5.0	5.0	5.0	5.0
Dicalcium phosphate	0.46	1.27	1.27	1.27	1.27
Limestone, pulverized	0.75	0.8	0.8	0.8	0.8
Salt	0.25	0.25	0.25	0.25	0.25
L-Lys·HCl	0.0	0.13	0.13	0.13	0.13
DL-Met	0.0	0.06	0.06	0.06	0.06
Choline chloride, 50%	0.1	0.1	0.1	0.1	0.1
Vitamin premix [2]	0.1	0.1	0.1	0.1	0.1
Mineral premix [3]	0.1	0.1	0.1	0.1	0.1
Total	100	100	100	100	100
Calculated chemical components					
ME, kcal/kg	3265	3265	3265	3265	3265
CP(%)	20.9	20.9	20.9	20.9	20.9
Ca(%)	0.7	0.7	0.7	0.7	0.7
P(%)	0.6	0.6	0.6	0.6	0.6
Total Lys%	1.24	1.24	1.24	1.24	1.24
Total Met%	0.4	0.4	0.4	0.4	0.4
Analyzed chemical components					
CP(%)	20.32	20.81	20.52	20.78	20.78
Ca(%)	0.68	0.69	0.69	0.71	0.70
P(%)	0.57	0.58	0.61	0.59	0.59

[1] TSFP: two-stage fermented feather meal-soybean meal product. [2] Vitamin supplied the following per kilogram of diet: vitamin A, 5000 IU; vitamin D_3, 1500 IU; vitamin E, 40 mg; vitamin K, 3 mg; vitamin B_1, 2.6 mg; vitamin B_{12}, 4 mg; niacin, 35 mg; pantothenic acid, 23 mg; vitamin B_2, 6 mg; vitamin B_6, 0.6 mg; niacin, 30 mg; pyridoxine, 1 mg; folic acid, 0.5 mg; biotin, 0.2 mg. [3] Mineral supplied the following per kilogram of diet: Fe ($FeSO_4 \cdot 7H_2O$, 20.09%Fe), 217 mg; Cu ($CuSO_4 \cdot 5H_2O$, 25.45%Cu), 125 mg; Mn ($MnSO_4 \cdot H_2O$, 32.49%Mn), 40 mg; Zn ($ZnSO_4$, 80.35%Zn), 110 mg; Se ($NaSeO_3$, 45.56%Se), 0.36 mg; Co ($CoSO_4 \cdot H_2O$, 32%Co), 0.7 mg; I (KI), 0.45 mg.

Table 2. Composition of experiment diets (as-fed basis) (trial 2).

Ingredients (%)	5% Fish Meal	TSFP, %		
		0	2.5	5
Corn meal	62.3	58.26	58.37	58.84
Soybean, full fat, cooked, 38% CP	6.0	10	10	10
Soybean oil	0.61	0.82	0.98	1.07
Soybean meal dehulled, solvent, 47.8% CP	21.8	24.8	22.4	19.8
Skim milk, dried	2.5	2.5	2.5	2.5
Fish meal (Peru), 65% CP	5.0	0.0	0.0	0.0
TSFP [1], 62% CP	0.0	0.0	2.5	5.0
Dicalcium phosphate	0.47	1.15	1.24	1.25
Limestone, pulverized	0.77	0.82	0.8	0.8
Salt	0.25	0.25	0.25	0.25
L-Lys·Hcl	0.0	0.04	0.1	0.12
DL-Met	0.0	0.06	0.06	0.07
Choline chloride, 50%	0.1	0.1	0.1	0.1
Vitamin premix [2]	0.1	0.1	0.1	0.1
Mineral premix [3]	0.1	0.1	0.1	0.1
Total	100	100	100	100
Calculated chemical components				
ME, kcal/kg	3265	3265	3265	3265
CP(%)	20.9	20.9	20.9	20.9
Ca(%)	0.7	0.7	0.7	0.7
P(%)	0.6	0.6	0.6	0.6
Lys%	1.22	1.22	1.22	1.22
Met%	0.4	0.4	0.4	0.4
Analyzed chemical components				
CP(%)	20.73	20.80	20.75	20.77
Ca(%)	0.69	0.68	0.67	0.70
P(%)	0.58	0.57	0.60	0.59

[1] TSFP: two-stage fermented feather meal-soybean meal product. [2] Vitamin supplied the following per kilogram of diet: vitamin A, 5,000 IU; vitamin D_3, 1,500 IU; vitamin E, 40 mg; vitamin K, 3 mg; vitamin B_1, 2.6 mg; vitamin B_{12}, 4 mg; niacin, 35 mg; pantothenic acid, 23 mg; vitamin B_2, 6 mg; vitamin B_6, 0.6 mg; niacin, 30 mg; pyridoxine, 1 mg; folic acid, 0.5 mg; biotin, 0.2 mg. [3] Mineral supplied the following per kilogram of diet: Fe ($FeSO_4 \cdot 7H_2O$, 20.09%Fe), 217 mg; Cu ($CuSO_4 \cdot 5H_2O$, 25.45%Cu), 125 mg; Mn ($MnSO_4 \cdot H_2O$, 32.49%Mn), 40 mg; Zn ($ZnSO_4$, 80.35%Zn), 110 mg; Se ($NaSeO_3$, 45.56%Se), 0.36 mg; Co ($CoSO_4 \cdot H_2O$, 32%Co), 0.7 mg; I (KI), 0.45 mg.

2.8. Statistical Analysis

The variables in physiochemical characterizations of TSFP, blood biochemistry, and immune characteristics were analyzed according to the following statistical model: $Yij = \mu + \tau i + eij$, where Yij represents the measured value on the i-th treatment in the j-th experimental unit; μ is the overall mean; τi is the effect of i-th treatment, and eij is the random error associated with Yij. For analyses of physiochemical characterizations and nutrient composition of TSFP were performed with 3 replicates (n = 3). For analyses of blood biochemistry and immune characteristics, individual pig of a total of 16 per group (n = 16) was considered as experimental units.

The variables in growth performance were analyzed according to the following statistical model: $Yij = \mu + \tau i + bj + eij$, where Yij represents the measured value of the experimental unit on the i-th treatment in the j-th block; μ is the overall mean; τi is the effect of i-th treatment; bj is the effect of j-th block, and eij is the random error associated with Yij. For analysis of growth performance, a single pen (n = 5) was considered as the experimental unit. For all analyses, the sex (gender) variable effect was excluded due to no significance between barrows and females.

The data were analyzed using the general linear model (GLM) procedure [26], and the groups were compared using a one-way ANOVA with Tukey post hoc test, where $p < 0.05$ indicated a statistically significant difference and $0.05 < p < 0.10$ indicated a tendency. Data

were presented as means ± pooled SEM. The polynomial contrasts were used to test the linear and quadratic effects of the increasing levels of TSFP. The scores in the physiological characterizations of FFSMP and the survival rate of growth performance were analyzed using the NPAR1WAY procedure [26]. The groups were compared using SAS® macro implementation of a multiple comparison test according to Elliott and Hynan [27], where $p < 0.05$ indicated a statistically significant difference.

3. Results

3.1. Trial 1, the Effects of Modified TSFP on the Growth Performance and Blood Biochemistry of Nursery Pigs

3.1.1. Physiochemical Analysis on TSFP with Different Fermentation Times at First Stage

Table 3 presents the effects of various times of 0 to 48 h in the first-stage fermentation on the physiochemical analysis of TSFP. The pH value, viscosity, odor, and γ-PGA content of TSFP were all increased in a time-dependent manner ($p < 0.05$) and reached the highest in the 48 h fermentation group ($p < 0.05$). The counts of Bacillus-like bacteria in each fermentation group were over 8 log CFU/g, which was higher than the control group ($p < 0.05$). The 40 h fermentation group had the highest counts ($p < 0.05$). In the second-stage fermentation, the pH value decreased, and the lowest was in the 32 h fermentation group ($p < 0.05$). The yeast counts in each group reached 7 log CFU/g at the end of the second-stage fermentation. Nevertheless, the 48 h fermentation group had the lowest counts ($p < 0.05$) as compared with the 32 h and 40 h fermentation groups. Moreover, the drying process reduced the Bacillus-like counts and did not detect any yeast counts. There was no significant difference in the nutritional composition of each treatment group ($p > 0.05$).

Table 3. Physiochemical characterizations of TSFP [1].

Items	1st Stage Fermented Time, h [2]				SEM	*p*-Value
	0	32	40	48		
First-stage fermentation						
pH	5.67 [d]	7.55 [c]	7.8 [b]	8.1 [a]	0.04	<0.0001
Bacillus-like, log CFU/g	1.04 [c]	8.35 [ab]	8.42 [a]	8.2 [b]	0.04	<0.0001
Viscosity (score)	1.2 [d]	3.0 [c]	3.8 [b]	4.5 [a]	-	<0.0001
Odor (score)	1.4 [d]	2.7 [c]	3.5 [b]	4.4 [a]	-	<0.0001
γ-PGA, %	ND [3]	2.1 [c]	3.8 [b]	5.3 [a]	0.02	<0.0001
Second-stage fermentation						
pH	-	5.13 [b]	5.78 [a]	5.85 [a]	0.03	<0.0001
Bacillus-like, log CFU/g	-	8.21	8.30	8.27	0.03	0.1174
Yeast-like, log CFU/g	-	7.94 [a]	7.86 [a]	7.54 [b]	0.02	<0.0001
Dry product						
pH	-	5.50 [b]	5.71 [a]	5.78 [a]	0.03	0.0008
Bacillus-like, log CFU/g	-	7.62 [a]	7.51 [b]	7.41 [b]	0.03	0.0031
Nutrient composition of dry product						
Moisture, %	9.50	9.61	9.48	9.45	0.05	0.1744
Crude ash,%/DM	4.63	4.62	4.65	4.63	0.09	0.9956
Crude protein, %/DM	62.0	62.5	62.3	62.7	0.80	0.9337
Gross energy, kcal/kg/DM	3165	3160	3148	3156	16.74	0.9048
Calcium, Ca %/DM	0.24	0.25	0.24	0.25	0.02	0.9722
Total phosphate, TP %/DM	0.55	0.56	0.55	0.54	0.02	0.9792

The data are given as mean, n = 3. [1] TSFP: two-stage fermented feather meal-soybean meal product. [2] TSFP was prepared with 0 h (unfermented group), 32 h, 40 h, or 48 h in the first-stage fermentation. [3] ND means not detected. [a–d] Means with the same letter in the row are not significantly different ($p < 0.05$).

3.1.2. Growth Performance

Table 4 presents the effect of modified TSFP on the growth performance of nursery pigs. At weeks 0 to 3 and 0 to 5, the BW, ADG, and FCR of the unfermented group were significantly poorer than the fermented groups ($p < 0.05$). At weeks 3 to 5, the ADG of 32-hr

and 48 h TSFP groups were better than the unfermented group ($p < 0.05$), and the FCR of the fish meal group was better than the unfermented group ($p < 0.05$).

Table 4. Effect of modified TSFP [1] on the growth performance of nursery pigs in trial 1.

Period, WK.	5% Fish Meal	1st Stage Fermented Time of 5% TSFP, h [2]				SEM	*p*-Value
		0	32	40	48		
Body weight (BW), kg							
0	8.85	8.88	8.84	8.90	8.92	0.18	0.9979
3	19.38 ^{ab}	18.68 ^{b}	19.96 ^{a}	20.03 ^{a}	20.07 ^{a}	0.31	0.0069
5	27.66 ^{ab}	26.50 ^{b}	28.59 ^{a}	28.52 ^{a}	28.69 ^{a}	0.41	0.0007
Average daily gain (ADG), kg							
0–3	0.50 ^{ab}	0.47 ^{b}	0.53 ^{a}	0.53 ^{a}	0.53 ^{a}	0.01	0.0001
3–5	0.59 ^{ab}	0.56 ^{b}	0.62 ^{a}	0.61 ^{ab}	0.62 ^{a}	0.01	0.0274
0–5	0.54 ^{ab}	0.50 ^{b}	0.56 ^{a}	0.56 ^{a}	0.57 ^{a}	0.01	<0.0001
Average daily feed intake (ADFI), kg							
0–3	0.75	0.71	0.74	0.74	0.73	0.01	0.2750
3–5	1.25	1.29	1.30	1.27	1.30	0.02	0.1142
0–5	0.95	0.94	0.96	0.95	0.96	0.01	0.6317
Feed conversion rate (FCR), ADFI/ADG							
0–3	1.50 ^{ab}	1.52 ^{a}	1.42 ^{b}	1.41 ^{b}	1.40 ^{b}	0.03	0.0211
3–5	2.05 ^{b}	2.32 ^{a}	2.15 ^{ab}	2.11 ^{ab}	2.18 ^{ab}	0.06	0.0111
0–5	1.77 ^{ab}	1.88 ^{a}	1.72 ^{b}	1.71 ^{b}	1.72 ^{b}	0.03	0.0002
Mortality (%)							
0–5	7.14	0.00	0.00	3.57	7.14	-	0.3939
Production efficiency factor (PEF), survival rate (%) × BW (kg))/(age (day) × FCR) × 100							
0–5	4193	4052	4804	4593	4394	205	0.0756

The data are given as mean, n = 5. [1] TSFP: two-stage fermented feather meal-soybean meal product. [2] TSFP was prepared with 0 h (unfermented group), 32 h, 40 h, or 48 h in the first-stage fermentation. [a,b] Means with the same letter in the row are not significantly different ($p < 0.05$).

3.1.3. Blood Biochemistry

Table 5 presents the effect of modified TSFP on the blood biochemistry of nursery pigs. The ALT, AST, LDH, TP, BUN, TG, CHOL, HDL-C, and LDL-C of the treatment groups were not significantly different ($p > 0.05$).

Table 5. Effects of modified TSFP [1] on the blood biochemistry of nursery pigs in trial 1.

Items [3]	5% Fish Meal	1st Stage Fermented Time of 5% TSFP, h [2]				SEM	*p*-Value
		0	32	40	48		
ALT (U/L)	37.2	37.2	36.0	37.0	38.4	1.42	0.8424
AST (U/L)	18.0	17.5	17.0	16.8	16.9	0.51	0.4542
ALP (U/L)	51.5	50.0	50.4	52.3	53.9	1.95	0.6427
LDH (U/L)	653	626	626	637	643	18.0	0.8185
TP (g/dL)	7.80	7.73	7.85	7.75	7.79	0.15	0.9854
BUN (U/L)	29.2	27.2	27.8	28.6	29.1	1.09	0.6529
TG (mg/dL)	50.8	50.5	50.1	51.9	51.7	1.56	0.9087
CHOL (mg/dL)	93.6	93.8	91.4	94.4	93.9	2.74	0.9468
HDL-C (mg/dL)	42.2	43.5	42.4	42.1	42.0	1.26	0.9217
LDL-C (mg/dL)	41.0	41.2	41.7	41.7	41.5	1.24	0.9933

The data are given as mean, n = 16. [1] TSFP: two-stage fermented feather meal-soybean meal product. [2] TSFP was prepared with 0 h (unfermented group), 32 h, 40 h, or 48 h in the first-stage fermentation. [3] ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; TP, total protein; BUN, blood urea nitrogen; TG, triglycerides; CHOL, cholesterol; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol.

3.2. Trial 2, Different Supplementation Levels of TSFP on the Growth Performance and Immune Characteristics of Nursery Pigs

3.2.1. Growth Performance

Table 6 presents the effects of different supplementation levels of TSFP on the growth performance of nursery pigs. As the dietary levels of TSFP increased to 5%, the ADG was significantly better than the 0% group ($p < 0.05$) at weeks 0 to 3, and the 5% TSFP group had better BW, ADG, FCR, and PEF than 0% group ($p < 0.05$) at weeks 0 to 5. In addition, a linear effect was observed on BW, ADG, ADFI, FCR, and PEF ($p < 0.05$) as the dietary level of TSFP increased.

Table 6. Effects of different supplementation levels of TSFP [1] on the growth performance of nursery pigs in trial 2.

Period, WK.	5% Fish Meal	TSFP, %			SEM	*p*-Value	Polynomial Contrasts	
		0	2.5	5			Linear	Quadratic
Body weight (BW), kg								
0	9.16	9.13	9.11	9.17	0.20	0.9963	0.7648	0.6170
3	19.31	18.48	19.35	19.68	0.34	0.0776	0.0112	0.8183
5	27.03 ^{ab}	25.90 ^{b}	27.21 ^{ab}	27.97 ^{a}	0.41	0.0068	0.0010	0.9936
Average daily gain (ADG), kg								
0–3	0.48 ^{ab}	0.45 ^{b}	0.49 ^{ab}	0.50 ^{a}	0.01	0.0176	0.0120	0.7243
3–5	0.55	0.53	0.56	0.59	0.02	0.0621	0.0079	0.7844
0–5	0.51 ^{ab}	0.48 ^{b}	0.52 ^{ab}	0.54 ^{a}	0.01	0.0023	0.0008	0.9756
Average daily feed intake (ADFI), kg								
0–3	0.73	0.73	0.74	0.75	0.01	0.0983	0.0150	0.8259
3–5	1.27	1.24	1.25	1.26	0.01	0.4419	0.9962	0.9132
0–5	0.94	0.93	0.94	0.96	0.01	0.1494	0.0711	0.6755
Feed conversion rate (FCR), ADFI/ADG								
0–3	1.54	1.65	1.55	1.52	0.04	0.1008	0.0674	0.8053
3–5	2.33	2.35	2.29	2.19	0.06	0.2447	0.0245	0.7149
0–5	1.88 ^{ab}	1.96 ^{a}	1.85 ^{ab}	1.79 ^{b}	0.04	0.0125	0.0014	0.8536
Mortality (%)								
0–5	10.71	14.29	7.14	3.57	-	0.5422	0.1853	0.8894
Production efficiency factor (PEF), survival rate (%) × BW (kg))/(age (day) × FCR) × 100								
0–5	3656 ^{ab}	3196 ^{b}	3919 ^{ab}	4354 ^{a}	256	0.0164	0.0049	0.9253

The data are given as mean, n = 5. [1] TSFP: two-stage fermented feather meal-soybean meal product. ^{a,b} Means with the same letter in the row are not significantly different ($p < 0.05$).

3.2.2. Immune Characteristics

Table 7 presents the effect of different levels of dietary TSFP on the immunity of nursery pigs. In ex vivo, dietary supplement 5% TSFP had higher potential activity on lipopolysaccharide (LPS) or concanavalin A (Con A)-induced lymphoblastogenesis as well as increasing the interferon-γ (IFN-γ) production compared with the fish meal group ($p < 0.05$). Moreover, the oxidative burst activity and the IgG production in the 5% TSFP group were higher than the 0% group and fish meal group ($p < 0.05$). There was no significant influence on the T cell populations among groups ($p > 0.05$). As the dietary level of TSFP increased, a linear effect was observed on LPS, IFN-γ, phagocytosis, oxidative burst activity, and IgG production ($p < 0.05$).

Table 7. Effects of different supplemented levels of TSFP [1] on the immunity of nursery pigs in trial 2.

Items [2]	5% Fish Meal	TSFP, %			SEM	*p*-Value	Polynomial Contrasts	
		0	2.5	5			Linear	Quadratic
Lymphoblastogenesis, specific fluorescence								
LPS	315 ^{b}	319 ^{ab}	338 ^{ab}	339 ^{a}	6.48	0.0135	0.0314	0.2578
CON A	195 ^{b}	200 ^{ab}	212 ^{ab}	217 ^{a}	5.39	0.0200	0.0508	0.6115
PMA/ION	267	269	278	288	7.39	0.1594	0.0980	0.9538
Cytokine, pg/mL								
Interferon-γ	108 ^{b}	111 ^{ab}	118 ^{ab}	119 ^{a}	2.92	0.0150	0.0449	0.3613
Mean fluorescence intensity								
Phagocytosis	74.0	73.4	76.3	78.2	1.80	0.2231	0.0462	0.8053
Oxidative burst	509 ^{bc}	492 ^{c}	546 ^{ab}	562 ^{a}	10.6	<0.0001	<0.0001	0.1493
Immunoglobulin, mg/dL								
IgA	1.27	1.27	1.30	1.32	0.04	0.7507	0.359	0.9689
IgM	1.68	1.67	1.73	1.74	0.04	0.5033	0.1991	0.7098
IgG	20.1 ^{b}	20.1 ^{b}	21.1 ^{ab}	22.0 ^{a}	0.49	0.0204	0.0153	0.9233
Blood T-lymphocyte subsets, %								
CD 3	71.7	73.7	72.4	71.2	1.47	0.6603	0.2526	0.9745
CD 4	22.6	24.1	24.1	22.7	0.84	0.4333	0.3013	0.5843
CD 8	36.6	38.0	41.3	39.7	2.32	0.2109	0.2097	0.2154
$CD3^{+}$ $CD4^{+}$	23.0	23.9	24.3	22.6	0.93	0.5632	0.3437	0.3809
$CD3^{+}$ $CD8^{+}$	32.2	31.1	34.3	33.5	1.56	0.4766	0.3177	0.3257
$CD4^{+}$ $CD8^{+}$	8.2	8.9	9.6	8.2	0.63	0.3375	0.3972	0.1686

The data are given as mean, n = 16. [1] TSFP: two-stage fermented feather meal-soybean meal product. [2] LPS (lipopolysaccharide); CON A (concanavalin A); PMA/ION (phorbal 12, 13 myristic acid/ionomycin). $^{a–c}$ Means with the same letter in the row are not significantly different ($p < 0.05$).

4. Discussion

4.1. Trial 1, the Effects of Modified TSFP on the Growth Performance and Blood Biochemistry of Nursery Pigs

4.1.1. Physiochemical Analysis

Bacillus spp. tends to grow in pH neutral environment [28]. During the fermentation process, the substrate protein is degraded and generates ammonia to increase the pH of the product [29]. The fermented substrate contained high protein and was utilized by *Bacillus* spp. to produce alkaline by-products in this experiment. During the first stage of fermentation, the pH increased from 5.6 to more than 7.5, and the highest pH value of 8.1 was attained at 48 h of fermentation. This was in agreement with the previous results reported by Huang et al. [18]. Moreover, it lowered the pH of the fermentation groups trending to below 5.85, along with increasing the counts of yeast when high-efficiency acid-producing strain Y10 was applied in the following 3-days second-stage fermentation. Previously, Huang et al. [18] demonstrated that using *B. coagulans* L12 anaerobic fermentation took 5 days to achieve similar pH as the current study. The current results prove that Y10 can efficiently produce acid and survive in the initially high pH environment. Moreover, the pH value did not change further when TSFP was subject to the final drying process. As the lactic acid produced by yeast does not volatile during the second-stage fermentation [19], the TSFP has also been kept in weakly acidic conditions.

The counts of Bacillus-like bacteria in each fermented group trend to rise from 1.04 to over 8.20 log CFU/g during the first-stage fermentation. This result indicated that *Bacillus* spp. could grow favorably by effectively using the substrate for fermentation. Bacillus strains have the property of forming endospores to resist high-temperature and low-pH environments [30]. Therefore, the fermentation at the second stage and final drying process has a limited impact on its survival and bacterial counts. After drying, the final counts remained above 7.41 log CFU/g. Similarly, Huang et al. [18] stated that the low pH of the two-stage fermentation process and the drying process did not affect the counts of Bacillus-like bacteria in the fermented product; their results were similar to this study. Along with the increase in fermented time, the counts of Bacillus-like bacteria in each fermentation group could reach more than 8 log CFU/g, reached a maximum at 40 hrs of fermentation, then decreased by 48 h of fermentation. Moreover, as the fermentation

time increased, the level of γ-PGA also increased, which was in attributing to the elevated viscosity of TSFP. Therefore, the air exposure was further isolated, causing the condition was not suitable for aerobic fermentation with Bacillus strains.

γ-PGA is a polymer formed by the dehydration condensation of the α-amino group and the α-carboxyl group of two glutamic acid molecules by the *Bacillus* strain. Generally, the higher content of γ-PGA resulted in a more viscous fermented product [31,32]. The γ-PGA content elevated as the fermentation time was extended, which led to the high viscosity. Furthermore, the contents of lipids and carbohydrates are responsible for the ferment odor. Due to the high protein content of the fermented substrate, protein degradation by microorganisms could generate ammonia and other unpleasant biological amines. It has been reported that the lysine, arginine, and tryptophan of the substrate were fermented by microorganisms to produce putrescine, cadaverine, and indole [33–35]. The odor and pH of the fermented product rose as the fermented time extended. It indicates that microorganisms continuously degrade the protein of the substrate to produce unpleasant odors such as ammonia and H_2S. To compare with Huang et al. [18], the TSFP production in this experiment can be shortened by 16 h in the first-stage process and 48 h in the second-stage process. Improvement has been achieved by greatly reducing the negative effects of fermentation time, viscosity, and odor.

4.1.2. Growth Performance

The TSFP produced by 32 h to 48 h in first-stage fermentation had improved the ADG and FCR of nursery pigs at 0–3 and 0–5 weeks. Fermentation of soybean meal by microorganisms can eliminate its anti-nutritional factors, thereby improving the utilization of nutrients and having better growth performance of piglets [3,4,36]. However, the study of fermented feather meal for piglet feed is relatively rare. Our previous study has reported that 5% of the two-stage fermented feather meal-soybean meal product from the aerobically fermented for 2 days with *B. subtilis* var. *natto* N21 (N21) at the first-stage and anaerobic fermentation with *B. coagulans* L12 (L12) for 5 days at the second stage into dietary fed to growing pigs resulted in growth performance as same as fish meal [22]. With different fermentation processes on mixed feather meal and soybean meal as fermented substrates, Huang et al. [18] subjected these substrates to 2-days aerobic fermentation with N21 and keratinase-producing strains *B. subtilis* Da2, Da15, and *B. amyloliquefaciens* Da6, Da16 at first-stage and 5-days anaerobic fermentation with L12 at the second stage. Then fed to the finishing pigs diet containing 2.5% or 5% of the two-stage fermented product had improved ADG and FCR of pigs compared with the control group and fish meal group. These results demonstrated that the two-stage fermented product can improve the growth performance of pigs. Using a combination of microbial strains, fermentation has more nutritive advantages in pig growth-promoting than the conventional one-stage aerobic fermentation with single-strain.

The TSFP takes 48 h in the first stage of aerobic fermentation, which often leads to excessive viscosity and odor in the fermentation process and is a drawback for application in the feed industry. Therefore, improvements in the first stage of aerobic fermentation were employed with the same fermentation strains adopted from Huang et al. [18] and investigated the aerobic fermentation time of 32, 40, and 48 h on the quality of TSFP. The 32-hr TSFP group had the highest counts of Bacillus-like bacteria among all groups, and its growth performance was no different from the 48-hr TSFP group during the entire experiment period, while it had less odor and viscosity. Additionally, the modification in the second-stage anaerobic fermentation with the Y10 strain adopted from Chen et al. [19] has resulted in faster acidification and a shorter fermentation time of only 72 hrs. The TSFP of the different fermentation times in the first-stage fermentation was supplemented at 5% in nursery piglet feed. The TSFP groups showed averagely improved BW, ADG, and FCR of nursery pigs at 0–3 weeks and 0–5 weeks compared with the unfermented group. The data supported that the modified TSFP can be developed in nursery pig dietary formulation with similar or even better growth performance than the conventional fish meal diet. Notably,

the ADG of 32 h and 48 h TSFP groups were significantly better than the unfermented group at weeks 3 to 5, while the FCR of the fish meal group only performed better than the control group. Apparently, high-quality fish meal still has a beneficial effect in improving the FCR of nursery pigs. Hence, it is still widely used by pig farmers. Nevertheless, the current two-stage fermentation provides effective bioprocessing to convert poorly digestible protein of feather meal into nutritive value-added easily digestible small molecules. In addition, the probiotics, unknown growth factors, and microbial metabolites of TSFP could be further beneficial in nursery pigs' growth.

4.1.3. Blood Biochemistry

Alanine aminotransferase is a liver-specific enzyme elevating in plasma when liver disease or injury occurs. AST is also widely distributed in various tissues. When liver cell injury, muscle injury, and myocardial necrosis, it will increase plasma AST levels. ALP mainly comes from the liver and bones. When plasma ALP level rises, it could be bile duct obstruction or other bone diseases. LDH is a common enzyme distributed in various tissues and can be used to indicate tissue damage [37]. Current analysis of clinical blood biochemistry, including ALT, AST, ALP, and LDH, was not significantly different among groups. The result supports the inclusion of 5% TSFP had no adverse effects on the overall health of pigs.

Moreover, there were no significant differences in the blood levels of TP, BUN, TG, CHOL, HDL-C, and LDL-C among the experimental groups. It indicates that TSFP supplementation had no negative effects on pig protein and lipid utilization and metabolism. It also shows that TSFP supplementation had no adverse impact on the clinical physiological assessments of piglets. Dietary supplementation of 5% TSFP can be free of safety concerns in nursery pigs.

4.2. Trial 2, Different Supplementation Levels of TSFP on the Growth Performance and Immune Characteristics of Nursery Pigs

4.2.1. Growth Performance

In trial 2, the TSFP produced by the improved fermentation process was fed to nursery pigs. The 5% TSFP group had improved ADG at 0–3 weeks and BW, FCR, and PEF at 0–5 weeks compared with the 0% group. The BW, ADG, FCR, and PEF of the 2.5% TSFP group were intermediate and could reach the same growth performance as the fish meal group. The ADG of each group was not significant at 3–5 weeks, but the 5% TSFP still showed a tendency of improvement compared with the control group ($p < 0.10$). The overall growth performance was similar to the outcomes of 5% TSFP added in trial 1. The reproducible outcomes show that the 2.5% TSFP inclusion can achieve the rather nutritive value as same as the 5% high-quality fish meal group. Moreover, as the level of TSFP supplementation increased, the mortality rate decreased from 14.29% (0% TSFP group) to 3.75% (5% TSFP group). Therefore, the 5% TSFP group improved PEF by 36% compared with the control group. Similarly, Huang et al. [18] used five mixed strains to facilitate high keratinase activity and strong feather decomposition ability. Adding 2.5% TSFP to finishing pigs improved ADG and FCR compared with the control group and fish meal group. Furthermore, dietary supplementation of 5% TSFP could improve ADG by 18.4% and FCR by 18.7% compared with those of the control group.

In summary, shortening aerobic fermentation time in the first stage from 48 h to 32 h and the anaerobic fermentation time in the second stage from 120 h to 72 h has provided far more economical production for the feed industry. Supplementation of TSFP at a level of 5% has shown a comparable or even better growth performance than 5% fish meal. It is visible to convert feather meal into a better nutrient utilization source with the current two-stage fermentation technology and to replace the costly fish meal in the nursery pig diet. It should be noted that the production scale of TSFP is not sufficient for industrial applications, but based on its effectiveness on piglets growth, it has the potential for commercial development.

4.2.2. Immune Characteristics

Immunity is divided into innate and adaptive immunities. Adaptive immunity is greatly dependent on lymphocyte proliferation in responding to antigens and resulting in memory lymphoblastic colonies for effective future defense. Lymphoblastogenesis activated by respective mitogens are used to assess acquired immunity in ex vivo [38]. Con A is a mitogen that activates T cell clonal proliferation via a non-specific binding to the T cell receptor. LPS is a glycolipid component of Gram-negative bacteria's outer membrane that can be mitogenic in stimulating B cell clonal proliferation. IFN-γ is a critical cytokine for innate immunity by enhancing the bactericidal effect of macrophages, also well known as the macrophage activating factor, and promoting cell-mediated immunity [39]. The current study showed that the LPS or Con A-induced lymphoblastogenesis, as well as the productions of IFN-γ and IgG of the 5% TSFP group, were significantly higher than the fish meal group. In addition, the oxygen burst capacity of the 5% TSFP group was significantly higher than the control group and fish meal group.

Studies have demonstrated that fermented soybean meal reduces inflammation and increases levels of immunoglobulin in piglets [4,36]. The current study is in agreement with Huang et al. [18], who indicated that adding 5% TSFP to the finishing pig diets could effectively improve the pig's immune functions. The improved immunity of piglets is determined by decreased mortality. The immune system has not maturely developed at weaning as compared to the finishing pigs, and stress in the weaning period often impairs immune functions. Moreover, the poor response to vaccinations and higher susceptibility to infectious diseases lead to high mortality. Upregulated immunity is promised to lower mortality in piglets. Supplementation of TSFP has shown a dose-dependently promoting lymphoblastogenesis and the production of IFN-γ and IgG. Moreover, TSFP supplementation at a level of 5% also significantly improved oxidative burst activity as compared with the fish meal group and the 0% group. Finally, it could be confirmed by the best PEF performance in the 5% TSFP group.

5. Conclusions

The current study demonstrated that the TSFP production process for 32 h aerobic fermentation at the first stage showed less odor and viscosity trend to a quick reduction in the pH to achieve the acidification purpose. When the dietary level of the TSFP supplement is up to 5%, it shows a beneficial effect on the growth performance and immunity of nursery pigs. Moreover, dietary TSFP has not affected the clinical blood biochemical value, and it can be used safely in nursery pig diets.

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