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Studies on improvement of feeding value of shrimp by-product meal for laying hens using dried-persimmon by-product

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干柿副産物を活用した採卵鶏用エビ副産物ミールの飼料価値改善に関する研究

By

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Chapter 1

General Introduction

Japan has been suffering from chronic feed shortages over recent decades, which is due to increasing demand for livestock products resulting from the westernization of food habits. The situation is getting similar also in developing countries in Southeast Asia, along with economic growth: this problem is particularly severe in Thailand, the author's home country, which is the leading producer of poultry products in Southeast Asia with the production performance of poultry egg and meat about 0.68 and 3.49 million metric tons, respectively (Scanes and Christensen, 2019; USDA, 2019). At the present, this problem is counter-measured by importing poultry feed ingredients, such as maize, milo, and soybean meal, because their infrastructure for feed production is not enough to supply feed resources required. Expanding the agricultural area to increase the self-sufficiency rate is not a good idea, because this would lead to decreased forest areas and hence global warming.

Therefore, the production of feed from waste will be highlighted: fundamentally, feed is to be made from by-products and low-quality food materials unsuitable for human consumption. In Japan, “eco-feed” made from surplus and wasted food, is tried to use to replace some parts of poultry feed, but this has not yet been a well-accepted method. In Thailand, agricultural by-products, such as dried cassava pulp (Khempaka *et al*., 2009), mulberry leaves (Lokaewmanee *et al*., 2009), and palm kernel cake (Chinajariyawong and Muangkeow, 2011), have been studied to use as feed ingredients for poultry, and some of them seem to be promising. Unfortunately, they are poor in protein, and hence cannot be used as alternatives to soybean meal which is a major and costly protein source in poultry feed. Consequently, a study on generating protein-rich by-products suitable for

poultry is required. In this context, shrimp by-product is one of the few promising candidates for that, because of the great amount of shrimp processing waste is generating in this country, against the background of the world's 6th largest shrimp production (BCG analysis, 2019).

Shrimp meal (SM), a dried by-product from shrimp processing industries, has a color-enhancing effect on fish and shrimps (Sandifer and Joseph, 1976; Torrissen *et al*., 1981; Kalinowski *et al*., 2007), and has commonly been used for aquaculture feed in many countries. Additionally, SM has the potential of being an alternative protein source for animal feed due to its high protein content: crude protein in SM ranged from 39% (Khempaka *et al*., 2006a) to 54% (Rahman and Koh, 2016a). Moreover, Khempaka *et al*. (2006a) and Rahman and Koh (2016a) reported that SM has greater essential amino acid contents, such as methionine, threonine, glycine and valine, than that of soybean meal. However, although the protein quality of SM has been recognized to be comparable to soybean meal, it has rarely been used for poultry feed, because of being difficult to be digested due to its high level of chitin, a low-digestible N-acetylated glucosamine polysaccharide (Austin *et al*., 1981; Khempaka *et al*., 2006a,b).

So far, several studies have been conducted to improve the digestibility of SM (Oduguwa *et al*., 1998; Fox *et al*., 1994; Septinova *et al*., 2010), and Rahman and Koh (2016b, 2016c, 2018) have been reported that part of chitin in SM was degraded by the treatment with diluted formic acid, and the treated SM can be contained in broiler and laying hen feed up to 10% and 15%, respectively, without any adverse effect, which is recognized to be an easy and inexpensive method, but not environmentally-friendly. Consequently, as an eco-friendly alternative, the preferred method would be the degradation of chitin in SM by waste products or unused by-products. In this connection, it is well-known that chitin is degraded by chitinase, and this enzyme exists in some plants (Wadsworth and Zikakis, 1984; Huynh *et al*., 1992; Broekaert *et al*., 1998). As a result of

literature search, it was known that high level of chitinase activity exists in persimmon (*Diospyros kaki*) fruits (Takii *et al*., 2010; Zhang *et al*., 2013). Fortunately, persimmon peel (PP) is generated abundantly as a by-product of dried persimmon production in the Southern Nagano area, where the Faculty of Agriculture of Shinshu University is located, which is Japan's largest dried persimmon producing area. Therefore, it is so easy to obtain a large amount of PP for experiments.

PP contains not only chitinase but also carotenoids having effects of egg yolk pigmentation (Takahashi *et al*., 2006; Oh *et al*., 2013) and antioxidation (Woodall *et al*., 1996; Sahin *et al*., 2006) and tannins having an antibacterial effect (Smith and Mackie, 2004; Jamroz *et al*., 2009; Liu *et al*., 2018). Therefore, this may also contribute to the egg-quality improvement and chicken health. However, tannins have a negative aspect, which is a digestion inhibition by means of binding of tannins to proteins (Horigome *et al*., 1988; Iji *et al*., 2004; Woyengo and Nyachoti, 2012).

In this chapter, it has been suggested that PP as a feed additive to SM diets has the advantages, such as promotion of chitin digestion, improvement of egg yolk color, and contribution of chicken health, and the disadvantage, such as reduction of protein digestibility. However, it is unknown whether PP affects advantageously or disadvantageously when PP is actually given to chickens because there have been few reports studying PP as a feed ingredient for poultry.

Consequently, in Chapter 2, chitinase activity, chemical composition, inclusive of tannin in PP, and *in vitro* digestibility of SM diets containing PP were measured to assess whether PP is worth using as a digestion promoting feed ingredient in SM diets. In Chapter 3, based on the results in Chapter 2, laying performance, nitrogen balance, and egg quality of laying hens fed with SM diets containing graded levels of PP were measured to discuss whether PP is effective to restore the decreased performance of laying hens given SM-supplemented diets. In addition, using ammonia excretion as an

index, the effect of tannin in PP on the populations of bad intestinal bacteria in chicken was discussed. In Chapter 4, ileal digestibility and chitinase activity, tannin concentration, and bacterial counts in the gastrointestinal tract in laying hens given SM diets containing PP were measured to verify the hypotheses obtained in Chapter 3. In Chapter 5, the practical use of SM diets in Japan and Thailand was discussed. As the PP product is not available in Thailand, alternatives should be found if SM is used practically in this country. In this connection, given that pepper and its by-products are abundantly available in Thailand, can these products be a candidate?

Chapter 2

Improvement in the *in vitro* Digestibility of Shrimp Meal by the Addition of Persimmon Peel

Abstract

The present study was conducted to analyze the chemical properties of PP and the *in vitro* digestibility of SM diets containing PP and to discuss whether PP can be used as a feed additive to promote the digestion of SM in chickens. The chemical composition and chitinase activity of dried PP was studied. SM diets containing PP were formulated according to the 4 by 6 factorial design: 4 levels of SM (0%, 10%, 15%, and 20%) × 6 levels of PP (0%, 2%, 4%, 6%, 8%, and 10%). The *in vitro* digestibility of dry matter (IVDMD), crude protein (IVCPD), and chitin (IVCD) were measured. PP was rich in nitrogen-free extract (NFE, about 74%) and tannin (2.8%), and the highest chitinase activity of PP was observed at pH 4.5. Approximately 50% of chitinase activity was also observed at acidic (3.0) and alkaline (8.0) pH. Its activity was slightly affected by pepsin treatment. IVDMD increased upon addition of up to 8% PP but decreased with an increase in the level of SM. When PP level was increased up to 6%, IVCPD in the group containing 0% SM changed slightly but increased in other groups containing SM. When PP level was more than 6%, IVCPD decreased in all the groups. IVCD increased dose-dependently with increasing levels of PP and decreased with increasing levels of SM. In conclusion, PP was rich in NFE, had high chitinase activity, and improved all digestibility parameters, such as IVDMD, IVCPD, and IVCD, in SM diets where the PP level was under 6%. Thus, the data obtained here suggest that up to 6% of PP can be safely included in SM diets as a digestion promoter.

Introduction

The practical use of SM seems to be difficult for chicken diets, because of the low digestibility of chitin present in SM (Khempaka *et al.*, 2006b). Rahman and Koh (2016c, 2018) reported that treatment with formic acid led to a reduction in the level of chitin present in SM and improved the digestibility of SM, and treated SM could replace soybean meal present in the laying hen diet by up to 15%. However, an eco-friendly method to enable organic poultry production needs to be considered. Using dietary chitinase may be interesting because chitinase is an enzyme that degrades chitin and is found in several plants. Among plant species, persimmon fruits have been reported to have high chitinase activity (Takii *et al*., 2010), and the peel of the astringent-type persimmon is a by-product obtained after processing of dried persimmon and is available in large amounts in production areas. Therefore, PP may be used for the degradation of chitin present in SM. However, a concern to use PP as a feed ingredient for chicken diets arises because of the high level of tannin present, which is a factor that could decrease the digestibility of animals (Iji *et al*., 2004; Mariscal-Landín *et al*., 2004). Nevertheless, not much information on the use of PP as a feed ingredient for chicken diets has been reported.

The purpose of the present study was to investigate the chemical properties of PP and the *in vitro* digestibility of SM diets containing PP and to discuss whether PP can be used as a digestion-promoting ingredient to improve the digestibility of SM.

Materials and Methods

Preparation of PP and SM

Sun-dried PP, a by-product from the process of dried persimmon made from Ichida-gaki, was purchased from three different places in Nagano Prefecture, Japan, such as Ijima-machi, Matsukawa-machi, and Iida city. The sun-dried PP was ground to pass through a 1.0-mm aperture for further processing. SM was prepared as follows: whiteleg shrimps (*Litopenaeus vannamei*) were purchased commercially from India, Japan, and Thailand in their frozen forms, thawed under running water, and then peeled. The peel wastes, such as heads and hulls, were dried in an oven at 55°C for 10 h, ground to pass through a 1.0-mm aperture, and then used as SM (Fig. II-1).

Chemical Analysis and Enzyme Assay

Proximate compositions, acid detergent fiber (ADF), and neutral detergent fiber (NDF) of SM and PP were analyzed according to the standard method (AOAC, 1990). Chitin was measured using the method described by Ghanem *et al.* (2003). Tannin was measured by the Folin-Denis method, a method which was used for persimmon tannin analysis in previous studies (Yamada *et al*., 2002; Park *et al*., 2004): briefly, the sample (5 mL), which was composed of soluble tannin extracted with 80% methanol and insoluble tannin extracted under reflux condition with 1% HCl in methanol at 80°C, was mixed with Folin-Denis reagent (5 mL), incubated at room temperature for 3 min, post which the sodium carbonate solution (5 mL) was added. Then, after 1 h, the absorbance was measured at 760 nm, and the amount of tannin was calculated as tannic acid equivalents. Chemical compositions of SM and PP are shown in Table II-1. Crude chitinase fraction of PP was obtained as follows (Koh and Iwamae, 2013): briefly, PP homogenized with the buffer having the desired pH was centrifuged, and the supernatant was salted out with 80% ammonium sulfate. The precipitate was dialyzed against the

extraction buffer and centrifuged. The supernatant was measured for protein content by CBB dye-binding method (Bradford, 1976) and then used as the crude chitinase fraction. Chitinase activity was measured as follows (Hirano, 1991): briefly, the reaction mixture composed of crude enzyme fraction and 1% (w/v) colloidal chitin was incubated at 37°C for 60 min. The reaction was stopped by adding 10% tungstate and 2/3 N sulfuric acid, post which the reaction mixture was centrifuged, and the reducing sugar in the supernatant was measured according to the modified Schales method (Imoto and Yagishita, 1971). One unit (U) of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of *N*-acetylglucosamine per min at 37°C.

Effects of pH and Pepsin Resistance of PP Chitinase

The effect of pH on PP chitinase was studied using 50 mM (final concentration) of citrate or sodium phosphate buffers ranging from pH 3.0 to 8.0 at 37°C for 60 min. Citrate buffer was used for pH 3.0-6.0 and sodium phosphate buffer for pH 6.5-8.0 (Zhang *et al*., 2013). The pepsin resistance of PP chitinase was studied using 50 mM glycine-HCl buffer at pH 2.0 containing 0, 5, and 10 mg pepsin/mL (10,000 U/mg protein, Nacalai Tesque Inc., Kyoto, Japan) at 37°C for 60 min (Esmaeilipour *et al*., 2012). Chitinase activity was measured, as mentioned earlier.

Experimental Diets and in vitro Digestibility Measurement

Twenty-four diets were formulated according to the 4 by 6 factorial design: 4 levels of SM (0%, 10%, 15%, and 20%) × 6 levels of PP (0%, 2%, 4%, 6%, 8%, and 10%) (Table II-2). PP and SM were added at the expenses of maize and soybean meal, respectively. Three diets were made in each combination because PP obtained from three different origins and SM obtained from a single origin (from Thailand) were used. SM obtained from Thailand was used in this digestibility study, because there was a slight difference in the nutritional value among the SM obtained from three different sources, and SM from Thailand was supplied abundantly. All diets were formulated to meet or

slightly exceed the nutrient requirement of laying hens recommended by the NRC (1994): chitin N was not included while calculating the CP content, due to the lack of evidence of chitin N utilization in birds.

In vitro digestibility was measured according to the method described by Saunders *et al*. (1973) with a slight modification: briefly, approximately 250 mg of each diet was suspended in 15 mL of 0.1 N HCl containing 1.5 mg pepsin (concentration was the same as that used in the pepsin resistance experiment) and gently shaken at 41°C for 3 h. After neutralization with 0.5 N NaOH, this was mixed with 7.5 mL of phosphate buffer (pH 8.0) containing pancreatin (3,220 U/g of amylase, 38,500 U/g of protease, and 1,600 U/g of lipase) (Nacalai Tesque Inc., Kyoto, Japan) and shaken at 41°C for 24 h. The mixture was then centrifuged, washed with distilled water, filtered, and dried. Dry matter (DM), CP, and chitin contents in the dried digesta were measured as mentioned earlier for measurement of *in vitro* digestibility of DM (IVDMD), CP (IVCPD), and chitin (IVCD).

Statistical Analysis

Statistical significance among the various treatments was determined using Tukey's multiple comparison tests at a significance level of 5%. The *in vitro* digestibility data were analyzed by the two-way ANOVA using the GLM procedure (SAS Institute, 2015).

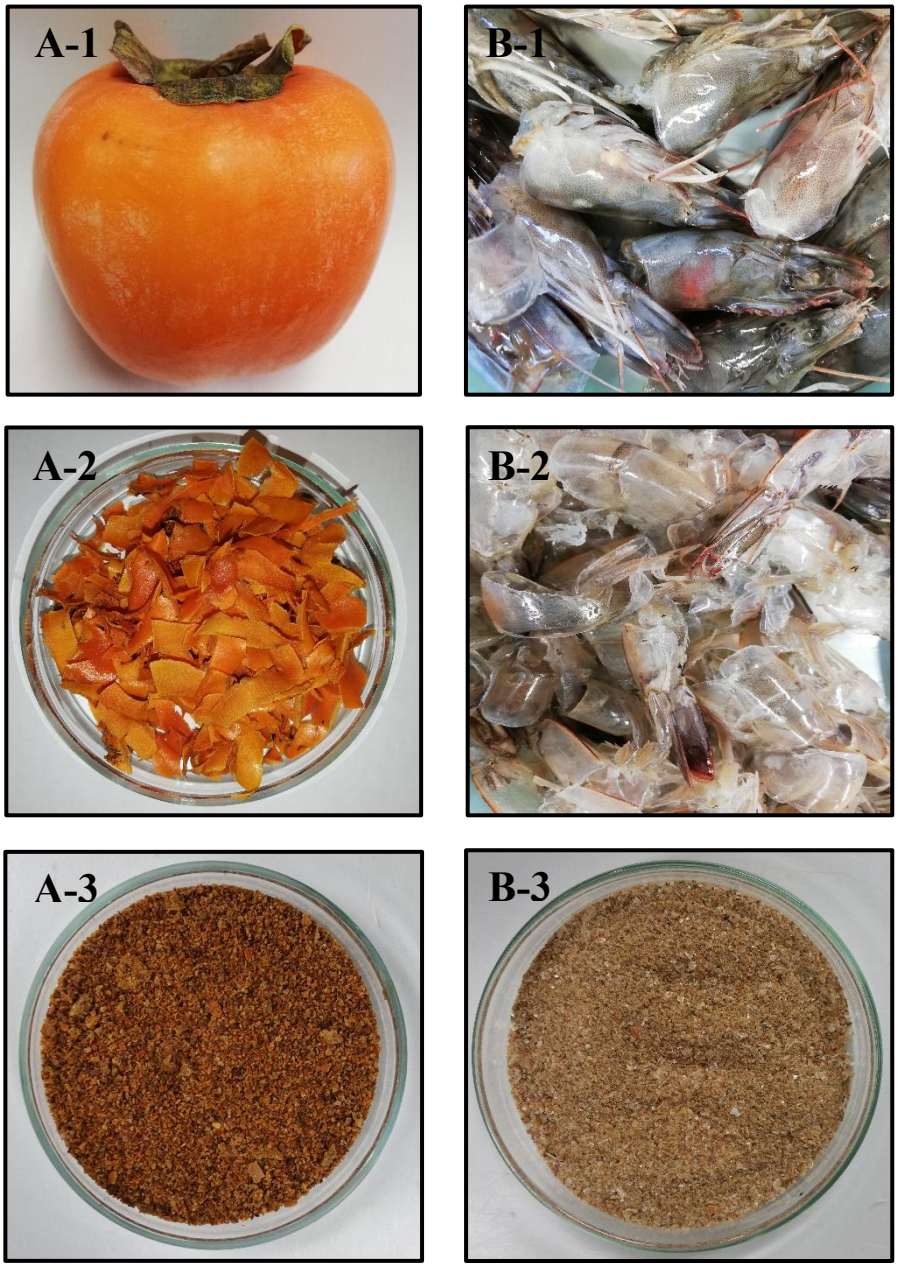


Fig. II-1. **Preparing persimmon peel and shrimp meal.** Astringent persimmon fruit 'Ichida-gaki' (A-1), persimmon peel (A-2), ground persimmon peel (A-3), shrimp heads (B-1), shrimp hulls (B-2), and shrimp meal (B-3).

Results

Chemical Properties of SM and PP

The major proximate composition of SM was CP, which was approximately 50% of the total composition. The second and third major components were crude ash and crude fiber, respectively. The other components were present at lower than 6% of the total composition. In SM, the value of NDF was 46%, which was more than two times greater than the value of ADF (Table II-1). Approximately 15% of chitin was present in SM. On the contrary, the major proximate composition of PP was NFE, which was approximately 74% of the total composition, and the second major component was crude fiber. The other components were present at lower than 5%. In PP, both NDF and ADF were present at approximately 30%, and tannin was present at approximately 3%. Maximum PP chitinase activity was obtained (about 1.30 U/mg of protein) at pH 4.5, and 50% activity was obtained at pH values ranging from 3.0 to 8.0 (Fig. II-2, panel A). PP chitinase activity at pH 2.0 containing no pepsin was about 0.58 U/mg of protein, which changed slightly in the presence of 5 mg/mL and 10 mg/mL pepsin in the buffer (Fig. II-2, panel B).

Effects of PP on Digestibility of SM Diets (Fig. II-3)

IVDMD in the group containing 0% SM was approximately 78% when no PP was present. IVDMD increased until the amount of PP added reached to 8% and then decreased. A similar pattern was found in other groups containing SM, but the values decreased overall with increasing amounts of SM. Results of two-way ANOVA showed that IVDMD was affected by both PP and SM significantly, but their interaction was not significant.

IVCPD in the group containing 0% SM was approximately 72% when no PP was present, and it changed slightly until the amount of PP added reached to 6% and then decreased. On the contrary, IVCPD in the other SM groups increased until the amount of

PP added reached to 6% and then decreased. With an exception of the group containing 0% SM, IVCPD decreased with an increase in the level of SM. Results of two-way ANOVA showed that IVCPD was affected by both PP and SM significantly, and their interaction was also significant.

IVCD in the groups containing no PP ranged from 18.8% (in the group containing 20% SM) to 24.9% (in the group containing 10% SM). As were the cases with IVDMD and IVCPD in the SM containing groups, IVCD increased with an increase in the level of PP and decreased with an increase in the amount of SM. However, no decrease was observed even when the amount of PP added was above 8%. Results of two-way ANOVA showed that IVCD was affected by both PP and SM significantly, but their interaction was not significant.

Table II-1. **Chemical compositions of shrimp meal and persimmon peel[1]**

Proximate composition, % of DM	Shrimp meal	Persimmon peel
Crude protein	51.3 ± 2.1	4.3 ± 0.3
Ether extract	5.6 ± 0.9	1.6 ± 0.1
Crude fiber	15.6 ± 0.7	17.2 ± 1.2
Crude ash	27.1 ± 1.9	3.3 ± 0.4
Nitrogen free extract (NFE)[2]	0.4 ± 0.1	73.6 ± 1.6
Chemical composition, % of DM		
Neutral detergent fiber (NDF)	46.0 ± 1.0	32.7 ± 2.1
Acid detergent fiber (ADF)	19.1 ± 0.3	30.7 ± 1.4
Chitin	14.7 ± 1.3	-
Tannin	-	2.8 ± 0.5

[1] The values of each parameter represent the mean ± standard error values with three observations (dry matter basis).

[2] NFE was calculated using the following equation: NFE, % = 100 − (crude protein, % + ether extract, % + crude fiber, % + crude ash, %).

Table II-2. **Composition and nutrient level of the basal diet (as-fed basis)**

Diets		Ingredients[1,2], g/kg							Chitin[3]
		Maize	PP	SBM	SM	Corn oil	$Ca_3(PO_4)_2$	$CaCO_3$	
SM0%	PP0%	503	0	323	0	35	35	85	0
	PP2%	483	20						
	PP4%	463	40						
	PP6%	443	60						
	PP8%	323	80						
	PP10%	403	100						
SM10%	PP0%	492	0	235	100	47	35	72	14
	PP2%	472	20						
	PP4%	452	40						
	PP6%	432	60						
	PP8%	412	80						
	PP10%	392	100						
SM15%	PP0%	489	0	190	150	53	30	69	21
	PP2%	469	20						
	PP4%	449	40						
	PP6%	429	60						
	PP8%	409	80						
	PP10%	389	100						
SM20%	PP0%	484	0	145	200	59	29	64	28
	PP2%	464	20						
	PP4%	444	40						
	PP6%	424	60						
	PP8%	404	80						
	PP10%	384	100						

[1] Vitamin-mineral premix provided with the following concentrations per kg of diet: vitamin A, 10,500 IU; vitamin D_3, 2,100 IU; vitamin E, 15 IU; thiamine, 10 mg; riboflavin, 7 mg; pantothenic acid, 15 mg; pyridoxine, 3 mg; niacin, 32 mg; choline chloride, 500 mg; folic acid, 0.6 mg; biotin, 0.1 mg; manganese, 75 mg; iron, 50 mg; zinc, 60 mg; copper, 5 mg; and iodine, 2 mg. This mixture was added to all diets at the level of 19 g/kg.

[2] All diets contained 176 g/kg of CP (analyzed value, as fed basis), 11.7 MJ/kg of ME, and 41 g/kg of Ca (calculated value, as fed basis). SM = shrimp meal, PP = persimmon peel, SBM = soybean meal.

[3] Measured value.

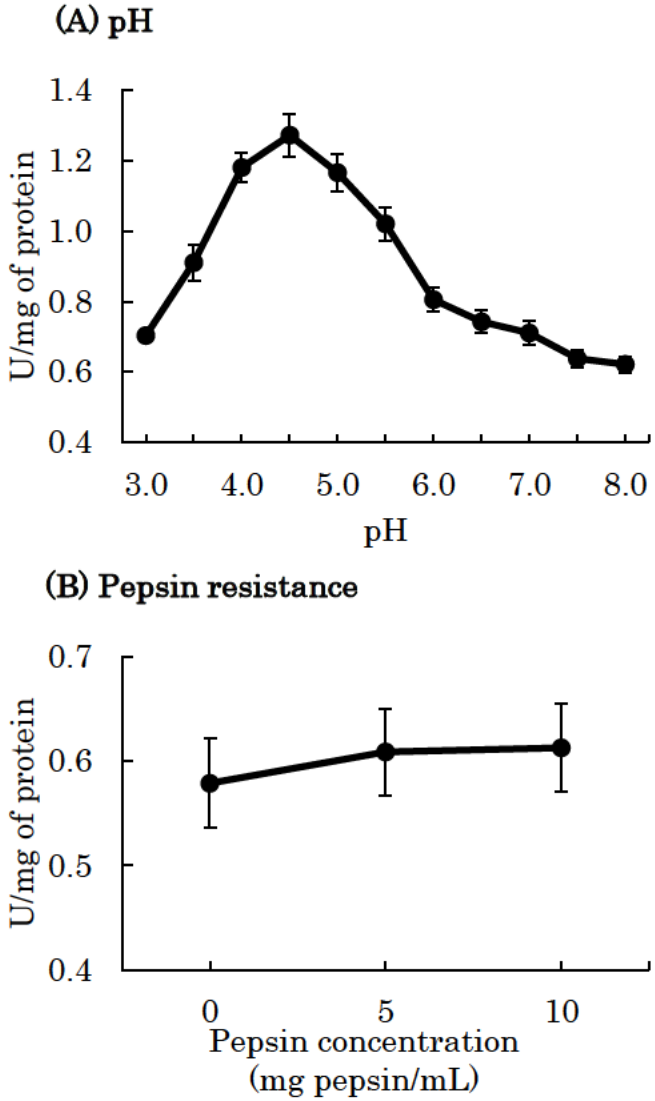
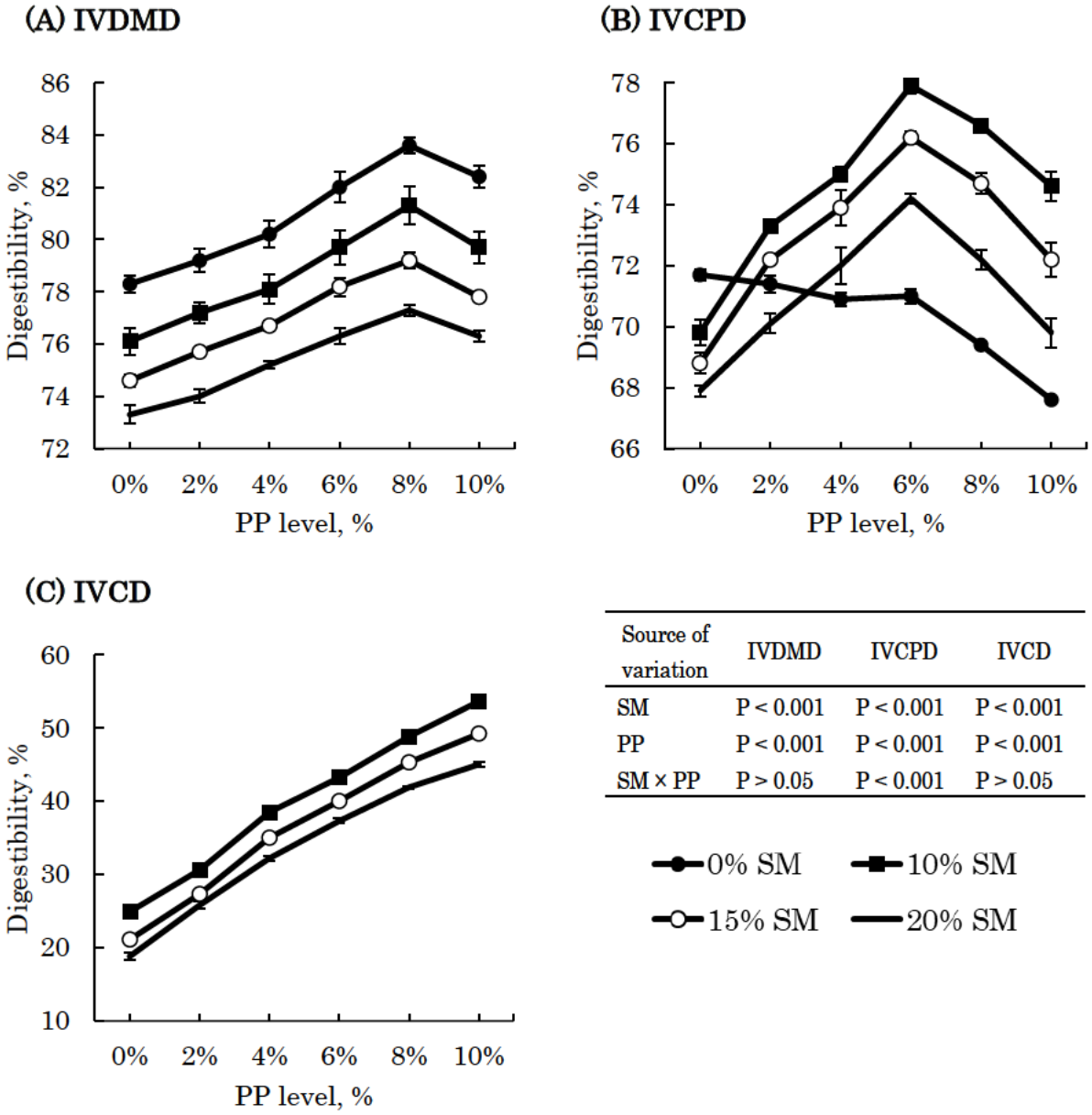


Fig. II-2. **pH behavior and pepsin resistance of persimmon peel chitinase.** Data represent mean ± SEM (n = 3). One unit (U) of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of *N*-acetylglucosamine per min at 37°C.



Source of variation	IVDMD	IVCPD	IVCD
SM	P < 0.001	P < 0.001	P < 0.001
PP	P < 0.001	P < 0.001	P < 0.001
SM × PP	P > 0.05	P < 0.001	P > 0.05

Fig. II-3. ***In vitro*** **digestibility of dry matter (IVDMD), crude protein (IVCPD), and chitin (IVCD) in shrimp meal diets containing persimmon peel.** PP = persimmon peel, SM = shrimp meal. Data represent mean ± SEM (n = 3).

Discussion

This is the first observation on the promoting effect of PP on the digestion of dietary SM.

SM used in the present study was rich in CP (about 50%), and the standard variation of each chemical composition in SM was very small, suggesting that the nutritional aspects of SM were similar among regions of different origins, such as India, Japan, and Thailand. PP was rich in NFE (about 74%), and thus it may be used as an energy source, although the presence of approximately 17% of crude fiber may be a concern. In PP, 2.8% of tannin was present, which may be an applicable level, because sorghum, widely used as a poultry feed ingredient, contains various levels of tannin, sometimes as high as 3.9% of tannin (Elkin *et al*., 1996). Maximum activity for PP chitinase was obtained at pH 4.5, and interestingly, relatively high activity (approximately 50%) was obtained at acidic (3.0) and alkaline (8.0) pH. Moreover, PP chitinase did not lose its activity even in the presence of buffer containing 10 mg/mL pepsin. Thus, PP chitinase is expected to function not only in the crop but also in the proventriculus and gizzard of chickens.

IVDMD in the group containing 0% SM increased with increasing levels of PP from 0% to 8%. However, it decreased when the amount of PP was increased to 10%. This pattern may be explained as follows: an increase in the IVDMD may be due to higher digestibility in PP than that in maize because PP in the group containing 0% SM was replaced with maize. In contrast, a decrease in the IVDMD may be due to an increase in the level of tannin. Tannins are well known to form complexes with proteins, starch, and digestive enzymes, and thus they reduce the nutritional values of food (Chung *et al*., 1998). Patterns of IVDMD in other groups with different percentages of SM were similar to that in the group containing 0% SM. No significant interaction between PP and SM levels was observed. Consequently, the pattern of IVDMD in all groups may be regulated

by a common mechanism. On the contrary, an overall decrease in the IVDMD with increasing levels of SM was observed, which may be due to poor digestibility of SM. The IVDMD of SM obtained from whiteleg shrimp was reported to be approximately 61.5% (Rahman and Koh, 2014).

When the amount of PP added was increased from 0% to 6%, the IVCPD in the group containing 0% SM changed slightly but increased in other groups containing SM. This could be due to complete digestion of the protein obtained from the non-SM origin with proteolytic enzymes, such as pepsin and protease in pancreatin, in the buffer, and limited or incomplete digestion of protein obtained from the SM origin by similar enzymes in the absence of PP chitinase. Therefore, PP may be a promising component to improve the digestibility of CP in SM. However, the IVCPD decreased not only in the group containing 0% SM but also in other groups containing SM, when the amount of PP added was increased to more than 6%. This could suggest that the digestibility of protein obtained from both the SM and non-SM origins were impaired by the tannin level in diets containing PP at 6% or more. Tannin levels decreasing *in vivo* digestibility of CP and amino acids are different among reports, such as 2.5% (Iji *et al*., 2004) and 0.41% (Woyengo and Nyachoti, 2012). However, the percentage of tannin reported in literature is higher than the percentage obtained in the present study: the tannin level in the diets containing 6% PP was 0.17%. This difference may be due to an increase in the pancreatic enzyme secretion after tannins bind to the digestive enzymes (Griffiths and Moseley, 1980).

About 20% of IVCD was recorded even in the group containing 0% PP, which indicates that there is another factor to digest chitin besides PP chitinase. Feed ingredients may not be the factor, because, compared with PP, very low chitinase activity was found in maize (0.19 U/mg of protein) (Huynh *et al*., 1992) and soybean (0.46×10^{-3} U/mg of protein) (Wadsworth and Zikakis, 1984): its activity in soybean meal may be lower than

that because of heating. High chitinase activity in SM may not be expected, because an increased level of SM leads to a decrease in IVCD. Moreover, pepsin in the buffer can be the factor because there is a report showing that this enzyme hydrolyzes not only protein but also chitin (Ilankovan *et al*., 2006). IVCD in all SM groups increased linearly with an increase in the level of PP, indicating that PP chitinase digested chitin in SM dose-dependently. However, unlike the results of IVDMD and IVCPD, IVCD did not decrease even when the amount of PP added reached 10%, suggesting that chitinase in PP was not affected by tannin. This might be true, because tannin-sensitive chitinase may not be able to function in persimmon fruits, as the role of plant chitinase is recognized as a defense mechanism against chitin-containing organisms (Zhang *et al*., 2013).

Conclusion

PP contained 2.8% of tannin, 73.6% of NFE, and high chitinase activity showing broad pH stability and pepsin resistance, suggesting that PP can be used as a feed ingredient promoting SM digestion, although it is predicted that tannin adversely affects digestibility when more than a certain amount of tannin is contained in SM diets. In addition, IVDMD and IVCPD of SM diets increased with increasing levels of PP, reached a maximum at PP inclusion levels of 8% and 6%, respectively, and then decreased, probably because of tannin. This suggests that PP level in SM diets maybe 6% when PP is used in order to promote SM digestion. However, in the case of *in vivo* study, the upper limit of PP level may probably be increased, because of a decreased adverse effect of tannin resulting from increased pancreatic enzyme secretion.

Chapter 3

Effects of Persimmon Peel on Laying Performance, Nitrogen Availability and Egg Quality in Laying Hens Provided with Shrimp Meal Diets

Abstract

In this chapter, to determine whether PP showing high chitinase activity could alleviate the detrimental dietary effects of chitin-rich SM, the laying performance, nitrogen (N) balance, and egg quality were assessed in laying hens provided with SM diets containing PP. The color and antioxidant properties of egg yolk were also examined, as anticipated, that these would be improved by providing SM and PP. Seventy-two laying hens (45 weeks of age) were allotted to one of the nine dietary treatments (eight hens each), namely three levels of SM (0%, 10%, and 15%) × three levels of PP (0%, 6%, and 8%), fed with the experimental diets over a period of 6 weeks. Hen-day egg production, feed intake, egg mass, feed conversion ratio, and N balance reduced with increasing levels of SM, whereas the reductions were recovered in a dose-dependent manner in response to increasing levels of PP; however, the SM0% treatment showed that PP exerted little effects. Notably, reductions in the Haugh unit and albumen height of eggs with increasing SM levels, and recovery by provision of increasing levels of dietary PP, were observed. Yolk color was improved by SM, although PP exerted little effect, whereas the antioxidant properties of yolk were enhanced by the inclusion of both SM and PP in diets. Furthermore, eggshell strength, weight, and thickness were enhanced with increasing levels of SM, whereas dietary PP had little effect on these parameters. Thus, it is suggested that PP can alleviate the negative effects of dietary SM and improve egg quality, without causing a reduction in laying performance, provided that the level of supplementary PP in diets is less than 8%. These findings accordingly indicate that PP is a promising feed constituent for laying hens fed with SM diets.

Introduction

The results in Chapter 2 revealed that PP having high chitinolytic activity improved the *in vitro* CP digestibility of SM-supplemented diets, reached a maximum when 6% of PP was added. These observations accordingly indicated that PP could serve as a valuable constituent in high-chitin feed. To the best of my knowledge, however, there have been no reports on use of PP for improvement of dietary chitin digestion in chickens.

Additionally, feeding dietary SM supplementation with PP may not be suitable for broilers, because an excessive level of calcium (Ca) in SM diets can depress the growth rate of broilers (Watkins *et al*., 1989; Gautier *et al*., 2017); moreover, high level of Ca in SM is unable to be reduced by the addition of PP. Consequently, dietary SM should be used for laying hens, as they need a high Ca level for the eggshell formation and maintaining eggshell quality (An *et al*., 2016).

The purpose of the present study was to determine the laying performance, nitrogen (N) balance, and egg quality in laying hens fed with SM diets containing PP, and to assess whether dietary PP alleviates the negative effects of SM. Additionally, the effects of dietary PP on the color and antioxidant properties of egg yolk were measured, as these may be affected by the high carotenoid (Takahashi *et al*., 2006) and tannin (Fitri *et al*., 2020) contents in PP. The levels of ammonia excretion were also measured as an indicator of the activity of unfavorable intestinal bacteria, as tannins can reduce the populations of these bacteria (Smith and Mackie, 2004; Jamroz *et al*., 2009).

Materials and Methods

The present study was conducted in accordance with the guidelines for regulation of animal experimentation of Shinshu University, Japan (Approval number 290085).

Test Products

Sun-dried PP, a by-product from a dried persimmon processing of Ichida-gaki, was purchased commercially from a local market in Nagano Prefecture, Japan, and sun-dried SM, composed of heads and hulls of the whiteleg shrimp (*L. vannamei*), was purchased commercially from Thailand. Samples of PP and SM were ground to pass through a 1.0-mm aperture and maintained at room temperature until used for analysis (Table III-1).

Hens, Diets, and Sampling Procedures

Seventy-two Lohmann LSL-Lite, laying hens (45 weeks of age), were divided into nine groups (each containing eight hens) with an average initial laying rate of 98%, and reared in individual cages under a 16L:8D light condition. Hens were allocated to one of the nine dietary treatments according to a 3 × 3 factorial arrangement i.e., three levels of SM (0%, 10%, and 15%) × three levels of PP (0%, 6%, and 8%) (Table III-2). SM and PP were added at the expenses of soybean meal and maize, respectively. Diets were formulated to meet or slightly exceed the nutrient requirements of laying hens recommended by the NRC (1994). However, chitin N was not included in the calculation of CP, as there is no evidence to indicate that birds utilize this source of N. The hens were fed with the experimental diets for 6 weeks, with the first week being used for adaptation and the subsequent five weeks for data collection. Both feed and water were provided *ad libitum*. The excreta of each hen were collected over the last 3 days of the second, fourth, and sixth weeks of the experimental period and stored at –20°C until used for analysis.

Laying Performance and Egg Quality

Egg production and feed intake (FI) were recorded daily. Hen-day egg production was calculated on a hen per day basis, and egg mass was calculated from egg production and egg weight, using the following equation: egg mass (g/hen) = (egg production × egg weight)/period (day). The feed conversion ratio (FCR) was calculated as the ratio of feed consumed to egg mass, and changes in body weight (BW) were calculated as the difference between the initial and final BWs. Egg weight, eggshell strength, Haugh unit, albumen height, and yolk color were measured daily using a digital egg tester (Nabel Co., Ltd., Kyoto, Japan). Eggshells were weighed after drying at 100°C for 2 h, and their thickness was measured using a micrometer (PK-1012CPX, Mitutoyo Corporation, Kanagawa, Japan).

Chemical Analysis

Samples of SM, PP, diets, and excreta were analyzed for proximate composition following standard methodology (AOAC, 1990). Chitin levels were measured using the method described by Ghanem *et al*. (2003). Chitinase activity was measured according to previously described methods in Chapter 2. Yolk lipid oxidation was determined weekly using the thiobarbituric acid reactive substance assay (TBARS), following the method described by Cherian *et al*. (1996). Briefly, fresh egg yolk (2 g) was homogenized with 18 mL of 3.86% perchloric acid and 50 µL of butylated hydroxytoluene. After filtration, the filtrate (2 mL) was mixed with 2 mL of 2 mM thiobarbituric acid and heated in a boiling water bath for 30 min, after which the absorbance was measured at 531 nm. Data were expressed in terms of nanograms of malondialdehyde (MDA) per gram of yolk. The amount of ammonia in fresh excreta was determined using the indophenol method (Scheiner, 1976).

Statistical Analysis

The data were subjected to two-way ANOVA using the GLM procedure (SAS Institute, 2015). When an interaction was significant, the Tukey Kramer test was performed, and when not significant, the data were combined over main effects and then subjected to the Tukey Kramer test. In all cases, a *P*-value < 0.05 was considered to be indicative of a statistically significant difference.

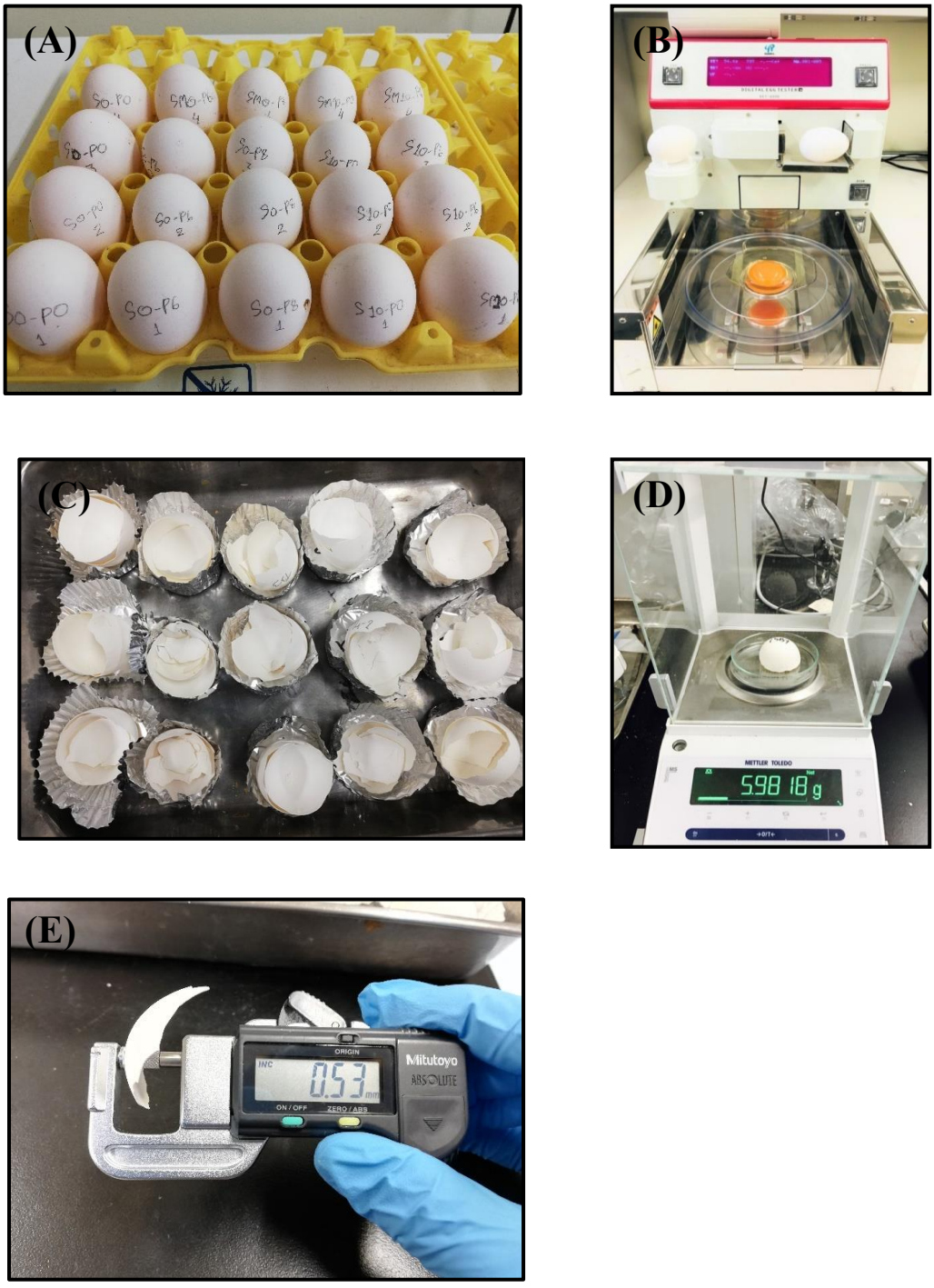


Fig. III-1. **Collection of eggs and analysis of egg quality.** Collected eggs (A), egg quality measurement (B), dried eggshell (C), eggshell weight measurement (D), and eggshell thickness measurement (E).

Table III-1. **Chemical composition of shrimp meal, persimmon peel, and maize**

Parameters, % of DM	Shrimp meal[1]	Persimmon peel[1]	Maize[2]
Crude protein	49.7	3.7	8.8
Ether extract	3.4	1.7	4.4
Crude fiber	16.9	15.2	2.0
Crude ash	29.4	3.1	1.4
Nitrogen free extract	0.6	76.3	83.4
Chitin	14.5	-	-
Chitinase activity, U/g protein	-	1,291	-

[1] The values of each parameter represent the mean values of triplicate analyses (in dry matter).

[2] Standard Table of Feed Composition in Japan (NARO, 2009).

Table III-2. **Composition and nutrient level of the experimental diets[1,2] (as-fed basis)**

Dietary groups		Ingredients, g/kg							Analyzed value	
		Maize	PP	Soybean meal	Corn oil	SM	Tricalcium phosphate	Calcium carbonate	Chitin, g/kg	Chitinase activity, U/g protein
0% SM	0% PP	266	0	162	34	0	27	42	0	0
	6% PP	190	60	171	41					77.5
	8% PP	164	80	175	43					103.9
10% SM	0% PP	253	0	76	47	100	22	33	15	0
	6% PP	175	60	87	54					77.5
	8% PP	151	80	89	56					103.9
15% SM	0% PP	248	0	32	53	150	19	29	22	0
	6% PP	171	60	42	60					77.4
	8% PP	147	80	44	62					103.7

[1] SM = shrimp meal; PP = persimmon peel.

[2] All diets contained 450 g/kg of commercial diet [crude protein (CP) > 17%, metabolizable energy (ME) > 2,850 kcal/kg, Nippon Formula Feed Mfg., Kanagawa, Japan] and 19 g/kg of vit-min premix providing the following nutrients per kg of diet: vitamin A, 10,000 IU; vitamin D_3, 2,100 IU; vitamin E, 15 IU; thiamine, 10 mg; riboflavin, 7 mg; pantothenic acid, 15 mg; pyridoxine, 3 mg; niacin, 32 mg; choline chloride, 500 mg; folic acid, 0.6 mg; biotin, 0.1 mg; manganese, 75 mg; iron, 50 mg; zinc, 60 mg; copper, 5 mg; and iodine, 2 mg.

[3] CP in all diets ranged from 17.0 to 17.2% (analyzed value), ME in all diets ranged from 2,901 to 2,903 kcal/kg, and Ca was 3.6% (calculated values).

Results

Laying Performance (Table III-3)

Hen-day egg production and FI were found to be significantly affected by both SM and PP, and their interaction. In the PP0% groups, hen-day egg production was 97.7% in the absence of SM but decreased significantly with increasing levels of SM ($P < 0.05$). However, with the exception of the SM0% group in which PP had no appreciable effect, the reduction in these values was dose-dependently recovered by increasing the level of dietary PP. A similar pattern was observed with respect to FI and egg mass. Although the interactive effect in the case of egg mass was not significant, the *P*-value was close to 0.05, and therefore the data were not combined. Given that there was no significant interaction, the FCR data were combined over main effects, as follows: the effect of SM levels (SM0% 1.95 ± 0.02^{b}, SM10% 1.96 ± 0.02^{b}, and SM15% 2.03 ± 0.02^{a}) and effect of PP levels (PP0% 2.04 ± 0.03^{a}, PP6% 1.96 ± 0.01^{b}, and PP8% 1.95 ± 0.02^{b}) ($^{a-b}$ mean values followed by different superscript letter differ significantly at the $P < 0.05$). These data indicated that FCR increased with increasing levels of SM and decreased with increasing levels of PP. In contrast, neither SM nor PP had a significant effect on the changes in BW.

N Balance (Table III-4)

All parameters relating to N balance were affected significantly by both SM and PP, as well as their interaction. The pattern of change in N intake was found to be similar to that of FI as shown before because all diets were isonitrogenous. N excretion in the PP0% groups increased significantly with increasing levels of SM ($P < 0.05$), but this increasing tendency became less evident with an increase in the level of PP; the exception being the SM0% group, in which N excretion was unaffected by PP. Furthermore, whereas there were significant reductions in N retention in the PP0% groups with increasing levels of

SM ($P < 0.05$), the reduction values were recovered dose-dependently by increasing the level of PP, with the SM0% group, in which PP had little effect, being the exception.

Egg and Eggshell Quality

The results of two-way ANOVA revealed that the effect of SM was significant on all parameters in Table III-5, except for egg weight and albumen height: the former was not significant, and the latter nearly reached significance ($P = 0.0619$), and the effect of PP was significant on Haugh unit, yolk color, and MDA: the effect on albumen height nearly reached significance ($P = 0.0507$). Additionally, there were no significant interactions in any of the parameters of egg and eggshell quality, and therefore each parameter was combined over the main effects (Table III-6). Egg weight was affected by neither SM nor PP. Haugh unit decreased significantly with increasing levels of SM and increased with increasing levels of PP ($P < 0.05$). Similarly, albumen height tended to decrease with increasing levels of SM and increased with increasing levels of PP. In contrast, there was a significant increase in yolk color with increasing levels of dietary SM ($P < 0.05$), although not PP. Moreover, there were reductions in the levels of MDA in response to inclusion of SM and PP in the diets ($P < 0.05$). Additionally, eggshell parameters significantly increased with increasing levels of SM ($P < 0.05$), whereas PP appeared to have little effect on eggshells.

Ammonia Excretion (Fig. III-3)

Ammonia excretion decreased significantly with increasing levels of PP ($P < 0.05$), whereas the effects of SM and the interaction between SM and PP were non-significant.

Table III-3. **Laying performance of laying hens fed with diets containing shrimp meal and persimmon peel[1,2]**

Dietary groups		Hen-day egg	Feed intake,	Egg mass,	FCR,	Body weight change,
SM, %	PP, %	production, %	g/hen/day	g/hen/day	g feed/g egg	g/hen/6 weeks
0	0	97.7 ± 0.6^{a}	114.5 ± 1.2^{ab}	58.2 ± 1.1^{a}	1.96 ± 0.03	40.6 ± 29.7
	6	97.3 ± 0.8^{a}	115.4 ± 1.0^{ab}	59.6 ± 0.9^{a}	1.94 ± 0.02	14.4 ± 29.0
	8	96.4 ± 1.0^{ab}	116.2 ± 1.2^{a}	59.3 ± 1.6^{a}	1.97 ± 0.04	-26.9 ± 26.1
10	0	87.2 ± 1.4^{cd}	107.4 ± 1.1^{cd}	52.9 ± 1.2^{bc}	2.04 ± 0.06	21.3 ± 16.2
	6	95.9 ± 0.7^{ab}	112.9 ± 1.4^{ab}	58.0 ± 0.6^{a}	1.95 ± 0.02	15.0 ± 22.9
	8	98.2 ± 0.8^{a}	116.1 ± 0.8^{a}	59.8 ± 1.2^{a}	1.90 ± 0.02	18.1 ± 31.7
15	0	82.8 ± 1.1^{d}	102.7 ± 1.3^{d}	50.1 ± 0.7^{c}	2.12 ± 0.02	-17.5 ± 28.4
	6	89.0 ± 0.9^{c}	110.2 ± 1.1^{bc}	55.4 ± 0.6^{ab}	2.00 ± 0.02	3.1 ± 23.7
	8	91.8 ± 1.3^{bc}	113.2 ± 0.8^{ab}	57.5 ± 1.1^{ab}	1.97 ± 0.03	4.4 ± 24.4
Source of variation		----------	----------	*P*-value	----------	----------
SM		0.0001	0.0001	0.0001	0.0081	0.7697
PP		0.0001	0.0001	0.0001	0.0020	0.8507
SM × PP		0.0001	0.0036	0.0624	0.1166	0.7971

[a-d] Mean values within the same column followed by different superscript letters are significantly different at the $P < 0.05$ level.

[1] SM = shrimp meal; PP = persimmon peel; FCR = feed conversion ratio.

[2] The values for each parameter represent mean ± standard error of eight observations.

Table III-4. **Nitrogen balance of laying hens fed with diets containing shrimp meal and persimmon peel[1,2]**

Dietary groups		N intake,	N excretion,	N retention,
SM, %	PP, %	g/bird/day	g/bird/day	g/bird/day
0	0	3.16 ± 0.03^{abc}	0.97 ± 0.04^{c}	2.19 ± 0.04^{ab}
	6	3.20 ± 0.03^{ab}	0.99 ± 0.03^{bc}	2.21 ± 0.03^{ab}
	8	3.25 ± 0.04^{a}	1.01 ± 0.04^{bc}	2.24 ± 0.05^{ab}
10	0	2.99 ± 0.03^{d}	1.11 ± 0.03^{ab}	1.88 ± 0.04^{d}
	6	3.17 ± 0.04^{abc}	0.92 ± 0.03^{cd}	2.25 ± 0.05^{ab}
	8	3.19 ± 0.02^{ab}	0.83 ± 0.02^{d}	2.36 ± 0.02^{a}
15	0	2.81 ± 0.04^{e}	1.19 ± 0.02^{a}	1.63 ± 0.04^{e}
	6	3.04 ± 0.03^{cd}	1.04 ± 0.02^{bc}	2.00 ± 0.04^{cd}
	8	3.10 ± 0.02^{bcd}	0.99 ± 0.03^{bc}	2.11 ± 0.03^{bc}
Source of variation		---------- *P*-value ----------		
SM		0.0001	0.0001	0.0001
PP		0.0001	0.0001	0.0001
SM × PP		0.0171	0.0001	0.0001

[a-e] Mean values within the same column followed by different superscript letters are significantly different at the $P < 0.05$ level.

[1] SM = shrimp meal; PP = persimmon peel; N = nitrogen.

[2] The values for each parameter represent mean ± standard error of eight observations.

Table III-5. **Egg and eggshell quality of laying hens fed with diets containing shrimp meal and persimmon peel**[1]

Dietary groups		Egg	Haugh	Albumen	Yolk	MDA[3],	Eggshell	Eggshell	Eggshell
SM, %	PP, %	weight[2], g	units[2]	height[2], mm	color[2]	ng/g yolk	strength[2], kgf/cm^2	weight[3], g	thickness[3], mm
0	0	60.0 ± 0.9	93.0 ± 0.9	8.6 ± 0.2	8.5 ± 0.1	44.7 ± 0.5	5.5 ± 0.1	6.2 ± 0.1	0.56 ± 0.005
	6	61.2 ± 0.7	93.3 ± 0.3	8.7 ± 0.2	8.5 ± 0.1	34.0 ± 0.4	5.6 ± 0.1	6.2 ± 0.0	0.55 ± 0.002
	8	61.5 ± 1.1	94.4 ± 0.9	9.0 ± 0.3	8.6 ± 0.1	32.9 ± 0.3	5.4 ± 0.1	6.1 ± 0.1	0.54 ± 0.005
10	0	60.7 ± 0.5	91.7 ± 1.2	8.3 ± 0.3	9.7 ± 0.2	31.2 ± 0.6	6.2 ± 0.1	7.0 ± 0.1	0.58 ± 0.019
	6	60.5 ± 0.6	93.0 ± 0.9	8.6 ± 0.2	9.9 ± 0.1	21.0 ± 0.5	5.9 ± 0.1	6.9 ± 0.1	0.56 ± 0.002
	8	60.8 ± 0.7	94.4 ± 0.9	9.0 ± 0.3	10.0 ± 0.2	20.0 ± 0.3	6.0 ± 0.2	7.0 ± 0.1	0.58 ± 0.020
15	0	60.6 ± 0.6	89.1 ± 1.0	8.1 ± 0.2	10.8 ± 0.1	28.8 ± 0.5	6.3 ± 0.2	7.1 ± 0.1	0.61 ± 0.027
	6	60.0 ± 0.5	91.8 ± 0.9	8.3 ± 0.2	11.3 ± 0.1	18.7 ± 0.3	6.3 ± 0.3	7.0 ± 0.2	0.60 ± 0.022
	8	60.4 ± 0.9	92.9 ± 0.8	8.5 ± 0.2	11.4 ± 0.2	17.6 ± 0.3	6.3 ± 0.2	7.0 ± 0.2	0.59 ± 0.021
Source of variation		---------- *P*-value ----------							
SM		0.3106	0.0067	0.0619	0.0001	0.0001	0.0001	0.0001	0.0025
PP		0.3632	0.0028	0.0507	0.0359	0.0001	0.7136	0.7252	0.4340
SM × PP		0.9197	0.6638	0.9315	0.6066	0.8824	0.8400	0.9663	0.9516

[1]SM = shrimp meal; PP = persimmon peel; MDA = malondialdehyde.

[2] The values for each parameter represent mean ± standard error of eight observations.

[3] The values for each parameter represent mean ± standard error of six observations.

Table III-6. **The results of the main effects on egg and eggshell quality in laying hens[1]**

Main effects	Egg weight[2], g	Haugh units[2]	Albumen height[2], mm	Yolk		Eggshell		
				Color[2]	MDA[3], ng/g yolk	Strength[2], kgf/cm^2	Weight[3], g	Thickness[3], mm
SM levels, %								
0	60.9 ± 0.53	93.6 ± 0.44^{a}	8.8 ± 0.12	8.5 ± 0.07^{c}	37.2 ± 1.31^{a}	5.5 ± 0.06^{b}	6.2 ± 0.05^{b}	0.55 ± 0.003^{b}
10	60.7 ± 0.36	93.0 ± 0.60^{ab}	8.6 ± 0.17	9.8 ± 0.11^{b}	24.1 ± 1.24^{b}	6.0 ± 0.09^{a}	6.9 ± 0.08^{a}	0.58 ± 0.009^{ab}
15	60.3 ± 0.39	91.3 ± 0.61^{b}	8.3 ± 0.11	11.2 ± 0.10^{a}	21.7 ± 1.24^{b}	6.3 ± 0.11^{a}	7.0 ± 0.10^{a}	0.60 ± 0.013^{a}
PP levels, %								
0	60.4 ± 0.40	91.3 ± 0.67^{b}	8.4 ± 0.14	9.7 ± 0.22	34.9 ± 1.73^{a}	6.0 ± 0.11	6.8 ± 0.11	0.58 ± 0.012
6	60.5 ± 0.34	92.7 ± 0.43^{ab}	8.5 ± 0.11	9.9 ± 0.25	24.6 ± 1.65^{b}	5.9 ± 0.11	6.7 ± 0.12	0.57 ± 0.009
8	60.9 ± 0.53	93.9 ± 0.51^{a}	8.9 ± 0.16	10.0 ± 0.26	23.5 ± 1.63^{b}	5.9 ± 0.12	6.7 ± 0.13	0.57 ± 0.011

[a-c] Mean values within the SM and PP groups followed by different superscript letters are significantly different at the $P < 0.05$ level.

[1] SM = shrimp meal; PP = persimmon peel; MDA = malondialdehyde.

[2] The values for each parameter represent mean ± standard error of 24 observations.

[3] The values for each parameter represent mean ± standard error of 18 observations.

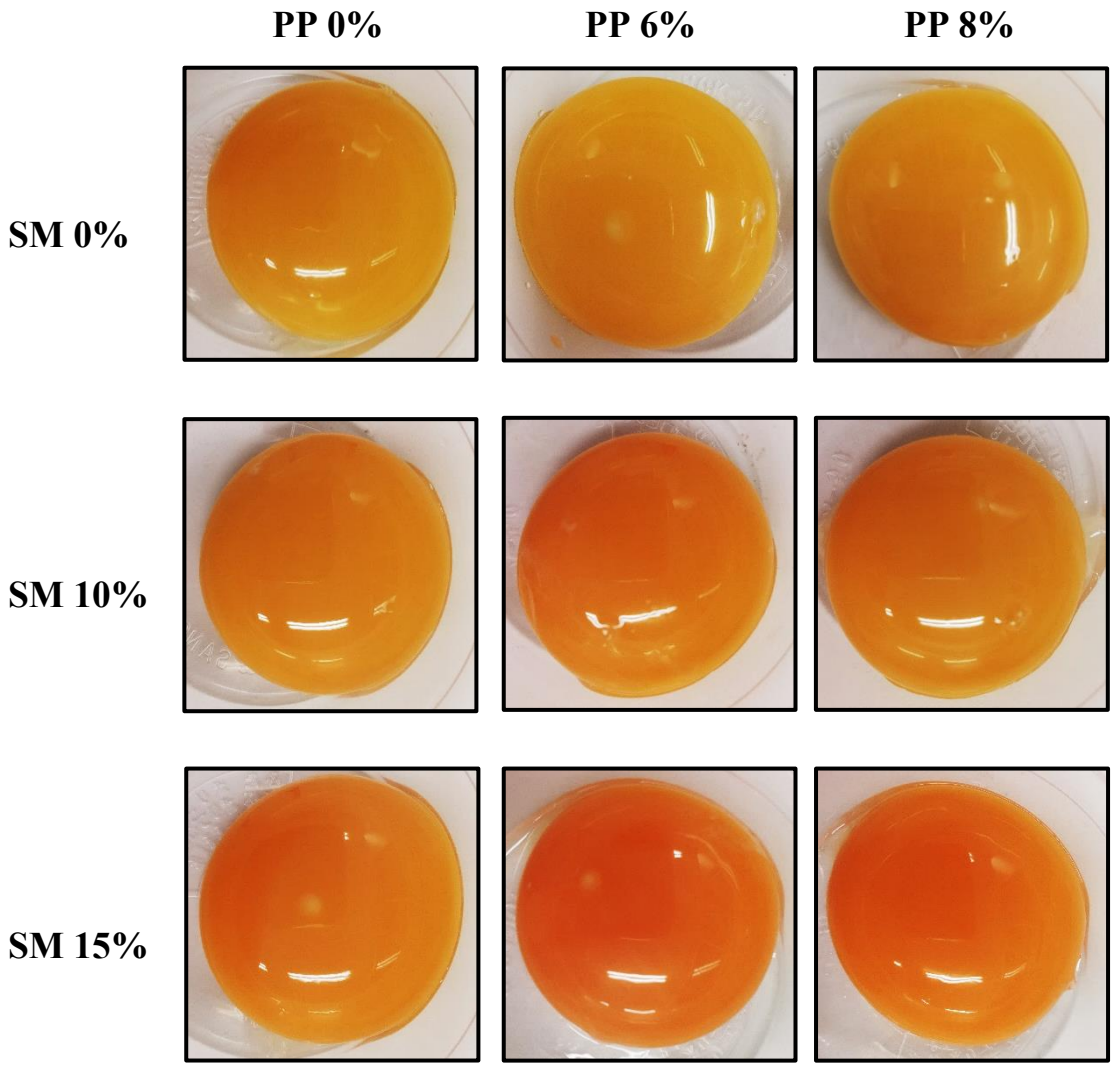


Fig. III-2. **Effect of dietary addition of shrimp meal (SM) and persimmon peel (PP) on egg yolk color.**

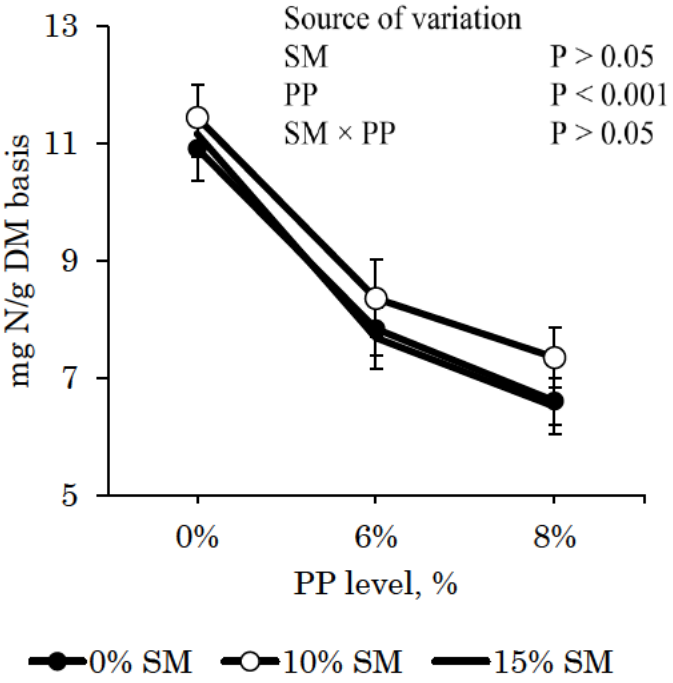


Fig III-3. **Ammonia excretion in laying hens fed with diets containing shrimp meal and persimmon peel.** SM = shrimp meal; PP = persimmon peel. Data represent mean ± standard error (n = 8).

Discussion

To the best of my knowledge, this study is the first to report on an improvement in the nutritional quality of SM diets of laying hens as a consequence of PP supplementation.

In the SM0% group, it was observed that PP had relatively little effect on hen-day egg production, FI, egg mass, and N balance. These findings lead to the following two hypotheses: (1) there is no significant difference in the nutritional values of PP and maize, the latter which was replaced with former in the experimental diets (Table III-1), and (2) PP has little effect on the digestibility and utilization of CP in laying hens as long as the supplemental level was at and below 8%. In this regard, the observations in Chapter 2 have been revealed that there was a reduction in the *in vitro* CP digestibility of an SM-free diet when supplemented with 8% PP, which it is suggested, could be attributable to the high levels of tannins. This discrepancy may be explained by an increase in the pancreatic enzyme secretion after tannins bind to the digestive enzymes (Griffiths and Moseley, 1980).

With respect to the PP0% group, it was observed reductions in hen-day egg production, FI, N intake, egg mass, and N retention, and increases in FCR and N excretion, in response to increasing levels of dietary SM, which is consistent with the findings reported by Rahman and Koh (2016c, 2018). These results thus tend to indicate that the negative effects of SM are attributable not only to a reduction in FI but also to an increase in FCR, namely, a reduction in nutritional utilization. In line with expectations, it was found that the negative effects of SM were alleviated by the addition of PP, with supplementation at the 8% level almost completely negating the detrimental effects of the SM10% diet, although this level of PP was not as effective in the case of the SM15% diet. This ameliorative effect of PP can be explained in terms of chitin digestion by PP chitinase, although this was not empirically determined. Dietary PP can potentially serve

as an alternative to formic-acid treatment, although as previously indicated, it would appear that PP contents in excess of 8% may be necessary in the case of diets comprising more than 10% SM. The development of such high-SM diets is desirable, given that this can not only enhance the utilization of SM, a by-product of shrimp-producing industries, but also enable a reduction in the use of soybean meal, an expensive imported CP source. However, although it may be feasible to increase the level of dietary SM by also increasing the content of PP, there may be an upper limit to beneficial PP supplementation, owing to the inhibitory effects of tannins on digestion.

Additionally, the present findings found reductions in the Haugh unit and albumen height of the eggs of hens fed with diets containing increasing levels of SM, with these effects being reversed by the supplementation of increasing levels of PP. Oh *et al*. (2013) consistently found that dietary PP improved the Haugh unit of eggs after 7 days of storage. Taking these observations into account, it would appear that although SM has a negative effect on albumen quality, the inclusion of PP in diets has a beneficial effect. It is suggested that the observed improvement in albumen quality can be attributed to the high ascorbic acid content of PP (Lee *et al*., 2006; Sorifa Akter *et al*., 2010), which has previously been reported to improve albumen quality (Keshavarz, 1996).

Consistent with the findings of Rahman and Koh (2018), the present study found that yolk color was enhanced by dietary supplementation with SM, whereas PP appeared to have relatively little effect. The coloring effect of SM may be attributable to the astaxanthin content of this product (Sowmya and Sachindra, 2012), which has previously been reported to enhance yolk color (Akiba *et al*., 2000; Anderson *et al*., 2008). Although PP is known to contain carotenoids that would be assumed to affect yolk coloring (Karunajeewa *et al*., 1984), there was no evidence for this under the conditions of the present study. Oh *et al*. (2013) have similarly reported that yolk color is little affected by dietary PP and PP ethanol extract. Additionally, it was also observed a significant

reduction in the MDA content of the eggs laid by hens fed with diets supplemented with SM and PP, which is consistent with the findings of previous studies indicating an increase in antioxidant activity attributable to carotenoid extracts obtained from shrimp processing waste (Sowmya and Sachindra, 2012), as well as from fresh and dried persimmon (Jung *et al*., 2005). Consequently, by feeding hens diets supplemented with SM and PP, it may possibly be to produce value-added eggs with high antioxidant activity.

Similarly consistent with the finding reported by Rahman and Koh (2018), the present findings found that supplementing hens diets with SM had the effects of enhancing eggshell strength, weight, and thickness, whereas comparatively, PP had little effect, as also reported by Oh *et al*. (2013). These observations thus tend to indicate that PP may not adversely affect eggshell quality, provided that the level of supplementation remains below 8%. It has also been reported that dietary polyphenols and tannins have the effects of reducing intestinal putrefactive products, including ammonia, via a reduction in the populations of unfavorable bacteria in the intestine (Terada *et al*., 1993; Hara *et al*., 1995). Therefore, it is assumed that the reduction in ammonia excretion observed in hens receiving PP-supplemented diets in the present study can be attributed to the inhibitory effects of PP tannins on such bacteria. However, given that ammonia in the excreta is not exclusively derived from the activities of intestinal bacteria, further studies will be necessary to clarify the effects of PP on the intestinal bacteria of laying hens.

Conclusion

PP could alleviate the detrimental effects of dietary SM on the performance of laying hens and could enhance the quality of eggs, including Haugh unit values and antioxidant properties, without causing a reduction in laying performance, as long as the levels of PP and SM supplementation did not exceed 8% and 10%, respectively. In this connection, although the supplemental level of 8% PP did not adversely affect the performance of laying hens, which suggests no reduction in CP digestibility in hens fed with 8% PP-supplemented diets, the effects of PP on nutritional digestibility of laying hens were not empirically examined. Additionally, it is plausible that dietary PP has the effect of reducing unfavorable intestinal bacteria, as PP could reduce ammonia excretion. Collectively, these findings indicate that PP can be effectively utilized as a quality improving constituent of SM-supplemented diets.

Chapter 4

Effects of Persimmon Peel on Ileal Digestibility and Bacterial Counts in the Gastrointestinal Tract of Laying Hens Provided with Shrimp Meal Diets

Abstract

In this chapter, to clarify whether PP can effectively serve as a digestion promoter for SM rich in chitin and the effect of PP on intestinal environment, *in vivo* digestibility, and chitinase activity, tannin content, and *Escherichia coli* count in each part of the gastrointestinal (GI) tract were measured in laying hens given diets containing SM and PP. Seventy-two laying hens (45 weeks of age) were allocated to nine dietary groups (eight hens each), namely three levels of SM (0%, 10%, and 15%) × three levels of PP (0%, 6%, and 8%), and fed for 6 weeks. On day 43 of the experiment, hens from each group were sacrificed to collect the digesta from each part of the GI tract. The deteriorations in ileal digestibilities of dry matter, crude protein, and chitin were observed when SM level increased, but these deteriorated parameters were dose-dependently recovered with increasing levels of PP. Chitinase activity in each intestinal content excepting ileal and cecal contents was increased by the addition of PP, particularly in the crop content, and the magnitude increased dose-dependently with increasing level of PP. Additionally, *E. coli* count was reduced by SM only in the crop but effectively reduced by PP in all parts of the GI tract, and tannin in PP may be the main factor of this reduction because tannin level increased toward the distal part of the GI tract. In conclusion, PP increased *in vivo* digestibility of SM and reduced detrimental bacteria in the GI tract of laying hens, and thus it is suggested that PP can potentially serve as a digestion promoter in laying hens given SM-supplemented diets and improve the intestinal environment of laying hens.

Introduction

The results in Chapter 3 suggest that the reduced FI, egg productivity, and N retention by feeding SM diets were recovered by the addition of PP to SM diets, and this recovery may be resulted from not only increased FI but also improved digestibility. In addition, the digestion inhibitors in PP, probably tannin, seemed to be less effective *in vivo* than *in vitro*. Thus, PP seemed to affect greatly the digestibility of SM diets, but there has been no direct information about *in vivo* digestibility of diets containing PP and SM in chickens.

In addition, the improvement in SM digestibility by PP seems to be involved with chitinase in PP, but it is also unclear where this digestion takes place in the digestive tract: there is a possibility that this enzyme can function throughout the intestinal tract due to its broad pH optimum and pepsin resistance.

Besides these, interestingly, the results in Chapter 3 also showed that PP has the reducing effect of ammonia excretion, suggesting that PP could suppress bad bacteria in the intestinal tract. The antibacterial components in PP may be tannin and/or chitinase because the former has already been reported to reduce intestinal bacteria (Smith and Mackie, 2004; Jamroz *et al*., 2009), and the latter has been found in the stomach of some animals (Suzuki *et al*., 2002; Tabata *et al*., 2018), including chickens, as a biodefense substance against bacteria-contaminated food.

The purpose of this chapter is to measure the *in vivo* digestibility, and chitinase activity, tannin content, and *E. coli* count in the gastrointestinal (GI) tract of laying hens given diets containing PP and SM, and to discuss the effectiveness of PP as a digestion promoter and the effect of PP on the intestinal environment.

Materials and Methods

The present study was conducted in accordance with the guidelines for regulation of animal experimentation of Shinshu University, Japan (Approval number 290085).

Test Products

Sun-dried PP, a by-product from a dried persimmon processing of Ichida-gaki, was purchased commercially from a local market in Nagano Prefecture, Japan, and sun-dried SM, composed of heads and hulls of whiteleg shrimp (*L. vannamei*), was purchased commercially from Thailand. Samples of PP and SM were ground to pass through a 1.0-mm screen and maintained at room temperature until used for analysis (Table IV-1).

Hens, Diets, and Sampling Procedures

Seventy-two Lohmann LSL-Lite, laying hens (45 weeks of age), were divided into nine dietary groups (each containing eight hens) and reared in individual cages under a 16L:8D light condition. Nine experimental diets were formulated according to a 3 × 3 factorial arrangement, namely three levels of SM (0%, 10%, and 15%) × three levels of PP (0%, 6%, and 8%) (Table IV-2). SM and PP were added at the expenses of soybean meal and maize, respectively. The diets were formulated to meet or slightly exceed the nutrient requirement of laying hens recommended by the NRC (1994); chitin N was not included in the calculation of CP, due to the lack of evidence of chitin N utilization in birds. All diets contained titanium dioxide (0.5%, TiO_2), as an indigestible marker. Hens were allocated to one of the nine diets for 6 weeks, with the first week being used for adaptation and the subsequent five weeks for data collection. Both feed and water were provided *ad libitum*. The excreta of each hen were collected over the last 3 days of the second, fourth, and sixth weeks of the experimental period and immediately stored at –20°C. Frozen excreta samples were then thawed, homogenized, dried, and ground for tannin analysis.

On day 43 of the experiment, after 12 h of fasting, hens were allowed to consume the feed for 1 h. Thirty minutes after feeding, four hens from each group were sacrificed to collect crop and gizzard digesta, and the remaining four hens from each group were sacrificed after 60 min to collect duodenal, jejunal, ileal, and cecal digesta (Fig. IV-1) (Chowdhury and Koh, 2018). The digesta from different parts of the GI tract were carefully collected into the sterile cups by standard aseptic technique and immediately determined bacterial counts. The remaining digesta were stored at –20ºC until further processing. Frozen digesta were then thawed, and fresh digesta were used for chitinase activity measurement, and the remaining digesta were dried at 40ºC in an oven and ground to pass through a 1.0-mm aperture for the measurements of ileal digestibilities of DM, CP, chitin, and tannin, including tannin content in each part of the GI tract.

Bacterial Enumeration

The bacterial counts were determined following the method described by Jin *et al*. (1998) with a slight modification: briefly, a volume containing 1 g of fresh digesta was mixed with 9 mL of sterile Dulbecco's phosphate-buffered saline (D-PBS, Nacalai Tesque Inc., Kyoto, Japan), subsequently homogenized, and serially diluted ten-fold using D-PBS solution. Then, 1 mL of each dilution was plated onto the Petrifilm™ *E. coli*/coliform count plate to isolate *E. coli* bacteria and then incubated at 35ºC for 24 h according to manufacturers' recommendations (3M interpretation guide, 2019). Colonies that occurred on the plate were enumerated, and data were expressed as the logarithm of colony-forming unit (log cfu) per gram of fresh digesta.

Chemical Analysis

Samples of SM, PP, diets, and ileal digesta were analyzed for proximate composition following standard methodology (AOAC, 1990). Chitin content was measured using the method described by Ghanem *et al*. (2003). Chitinase activity was measured following the methods as described in Chapter 2. Tannins of persimmon after drying are mainly

insoluble tannins (Hamauzu and Suwannachot, 2019) because soluble tannins in astringent persimmon fruits are converted into insoluble tannins via dehydration processes (Matsumura *et al*., 2016). Therefore, the amounts of tannin in dried PP, diets, digesta, and excreta were measured by using HCl-butanol assay, the insoluble tannin determination method, described by Brenes *et al*. (2008). Briefly, the sample was extracted with 50% methanol and 70% acetone (50 mL/g of sample, shaking for 60 min each). After centrifugation, the residue of the above extract was treated with HCl-butanol (5 mL) at 100°C for 3 h (Reed *et al*., 1982), and the absorbance was read at 550 nm. Persimmon tannin was used as a standard. The concentrations of TiO_2 in diets and ileal digesta were determined using the method described by Short *et al*. (1996).

Calculation and Statistical Analysis

The following equation was used to calculate ileal digestibility of nutrients (Meng *et al*., 2005):

Ileal digestibility (%) = $\{1 - [(TiO_{2\%\ diet}/TiO_{2\%\ digesta}) \times (Nutrient_{\%\ digesta}/Nutrient_{\%\ diet})]\} \times 100$.

The data were subjected to two-way ANOVA using the GLM procedure (SAS Institute, 2015): when the interaction was significant, the Tukey Kramer test was performed, and when not significant, data were combined over the main effects and then subjected to the Tukey Kramer test. In all cases, statistical difference was accepted when $P < 0.05$.

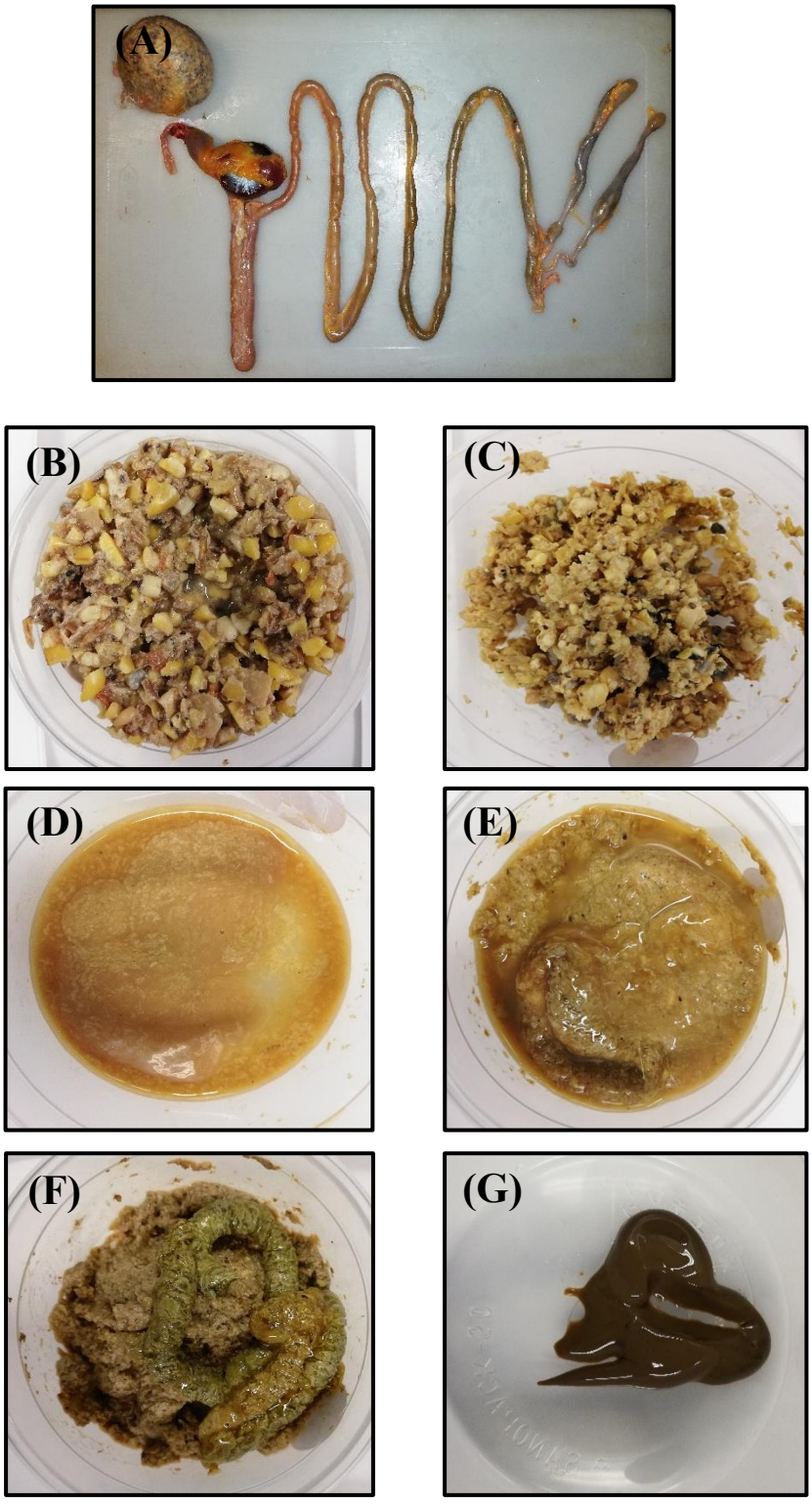


Fig. IV-1. **The gastrointestinal (GI) tract of chickens and the digesta obtained from various parts of the GI tract.** The GI tract (A), crop digesta (B), gizzard digesta (C), duodenal digesta (D), jejunal digesta (E), ileal digesta (F), and cecal digesta (G).

Table IV-1. **Chemical composition of shrimp meal and persimmon peel**

Chemical composition, % of DM[1]	Shrimp meal	Persimmon peel
Crude protein	49.7	3.7
Ether extract	3.4	1.7
Crude fiber	16.9	15.2
Crude ash	29.4	3.1
Nitrogen free extract	0.6	76.3
Chitin	14.5	-
Tannin	-	3.3
Chitinase activity, U/g protein	-	1,291

[1] The values of each parameter represent the mean values of triplicate analyses (in dry matter).

Table IV-2. **Composition and nutrient level of the experimental diets[1,2,3] (as-fed basis)**

Dietary groups		Ingredients, g/kg							Analyzed value		
		Maize	PP	Soybean meal	Corn oil	SM	$Ca_3(PO_4)_2$	$CaCO_3$	Chitin, g/kg	Tannin, g/kg	Chitinase activity, U/g protein
0% SM	0% PP	266	0	162	34	0	27	42	0	0	0
	6% PP	190	60	171	41					2.0	77.5
	8% PP	164	80	175	43					2.6	103.9
10% SM	0% PP	253	0	76	47	100	22	33	15	0	0
	6% PP	175	60	87	54					2.0	77.5
	8% PP	151	80	89	56					2.6	103.9
15% SM	0% PP	248	0	32	53	150	19	29	22	0	0
	6% PP	171	60	42	60					2.0	77.4
	8% PP	147	80	44	62					2.6	103.7

[1] SM = shrimp meal; PP = persimmon peel.

[2] All diets contained 450 g/kg of commercial diet [crude protein (CP) > 17%, metabolizable energy (ME) > 2,850 kcal/kg, Nippon Formula Feed Mfg., Kanagawa, Japan] and 19 g/kg of vit-min premix providing the following nutrients per kg of diet: vitamin A, 10,000 IU; vitamin D_3, 2,100 IU; vitamin E, 15 IU; thiamine, 10 mg; riboflavin, 7 mg; pantothenic acid, 15 mg; pyridoxine, 3 mg; niacin, 32 mg; choline chloride, 500 mg; folic acid, 0.6 mg; biotin, 0.1 mg; manganese, 75 mg; iron, 50 mg; zinc, 60 mg; copper, 5 mg; and iodine, 2 mg.

[3] CP in all diets ranged from 17.0 to 17.2% (analyzed value), ME in all diets ranged from 2,901 to 2,903 kcal/kg, and Ca was 3.6% (calculated values).

Results

Ileal Digestibility (Fig. IV-2)

The results of two-way ANOVA showed that all ileal digestibility parameters were significantly affected by both SM and PP, except for ileal tannin digestibility in which this value was affected by only PP, and the interaction between SM and PP was significant only in ileal CP digestibility. Ileal DM digestibility significantly increased with increasing levels of PP, irrespective of SM levels in diets, but overall decreased ($P < 0.01$) with increasing levels of SM. In the SM0% groups, ileal CP digestibility was 73.5% in the absence of PP and hardly affected with increasing levels of PP. Excepting the SM0% groups, ileal CP digestibility significantly increased with increasing levels of PP but significantly decreased with increasing levels of SM. Ileal chitin digestibility decreased with increasing levels of SM but increased with increasing levels of PP dose-dependently. Ileal tannin digestibility approximately ranged from 29 to 32% in hens fed with diets containing 6% and 8% of PP.

Chitinase Activity (Table IV-3)

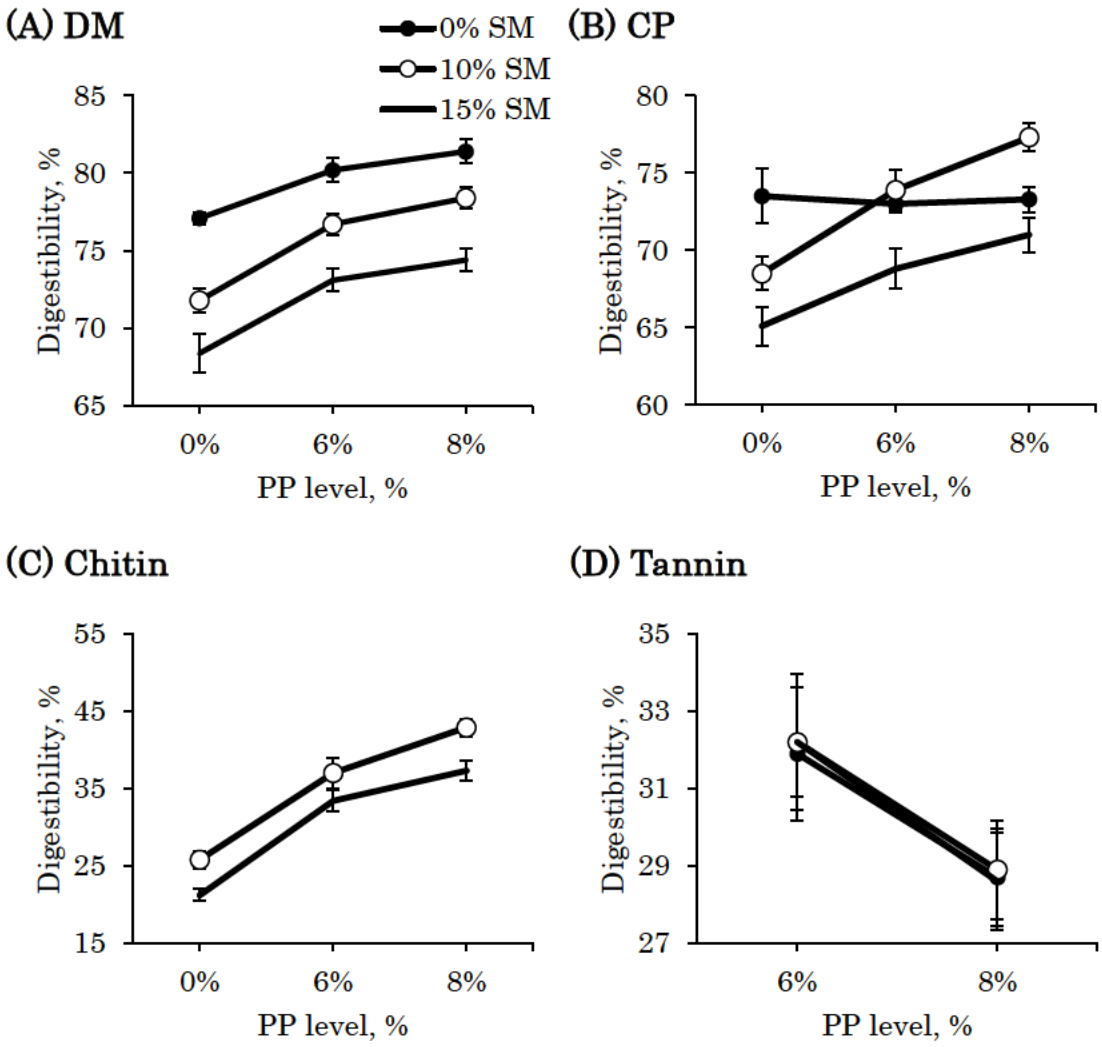
Chitinase activity was significantly affected by only PP in each part of the GI tract except the gizzard in which the value was affected by neither SM nor PP, and no interactions between SM and PP were observed, and therefore the data of chitinase activity were combined over the main effects. Chitinase activity in the PP0% group was not detected in the diets and crop content, but this value increased significantly in the gizzard content and then decreased below the duodenum and was not detected in the ileal and cecal contents. The addition of PP to diets increased chitinase activity in each intestinal content, particularly in the crop content, and the magnitude increased dose-dependently with increasing level of PP. The changing pattern of chitinase activity of PP groups in each part of the GI tract showed the same pattern with PP0% group.

Bacterial Counts (Fig. IV-3)

E. coli counts were significantly decreased by PP in all parts of the GI tract and decreased by SM only in the crop, and the interaction between SM and PP was not significant.

Tannin Content (Table IV-4)

Tannin content was significantly affected by both SM and PP in all parts of the GI tract and excreta, except for the crop and gizzard in which the values were affected only by PP, and the interaction between SM and PP was significant only in the cecum. SM and PP affected tannin content in the GI tract with the same pattern, which is the amount of tannin in PP-supplemented diets slightly changed in the crop and gizzard, and then increased gradually along the intestinal tract until in the excreta.



Source of variation	DM	CP	Chitin	Tannin
SM	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P > 0.05$
PP	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
SM × PP	$P > 0.05$	$P < 0.001$	$P > 0.05$	$P > 0.05$

Fig. IV-2. **Ileal digestibilities of dry matter (DM), crude protein (CP), chitin, and tannin of laying hens fed with diets containing shrimp meal and persimmon peel.** SM = shrimp meal, PP = persimmon peel. Data represent mean ± standard error ($n = 4$).

Table IV-3. **Chitinase activity (U/g protein) of the diets and digesta obtained from different parts of the gastrointestinal tract in laying hens[1,2,3]**

Main effects	Diets	The gastrointestinal parts			
		Crop	Gizzard	Duodenum	Jejunum
Time after feeding, min		30	30	60	60
SM level, %					
0	60.7	61.7 ± 13.7^{A}	2,361.6 ± 49.1^{B}	111.1 ± 11.6^{A}	35.4 ± 5.6^{A}
10	60.5	58.4 ± 13.0^{A}	2,338.6 ± 117.2^{B}	114.7 ± 11.5^{A}	34.3 ± 5.6^{A}
15	60.6	60.2 ± 13.4^{A}	2,282.0 ± 54.3^{B}	115.2 ± 10.6^{A}	35.6 ± 6.1^{A}
PP level, %					
0	0.0	0.0^{Aa}	2,276.6 ± 67.8^{B}	64.2 ± 3.4^{Aa}	9.7 ± 0.4^{Aa}
6	77.5	77.1 ± 2.2^{Ab}	2,339.2 ± 103.6^{B}	127.0 ± 2.6^{Ab}	40.8 ± 1.1^{Ab}
8	103.7	103.1 ± 2.8^{Ac}	2,366.4 ± 60.9^{B}	149.8 ± 1.8^{Ac}	54.8 ± 1.2^{Ac}
Source of variation		---------- *P*-value ----------			
SM		0.5870	0.8023	0.5165	0.6215
PP		0.0001	0.7575	0.0001	0.0001
SM × PP		0.9209	0.9904	0.5925	0.3907

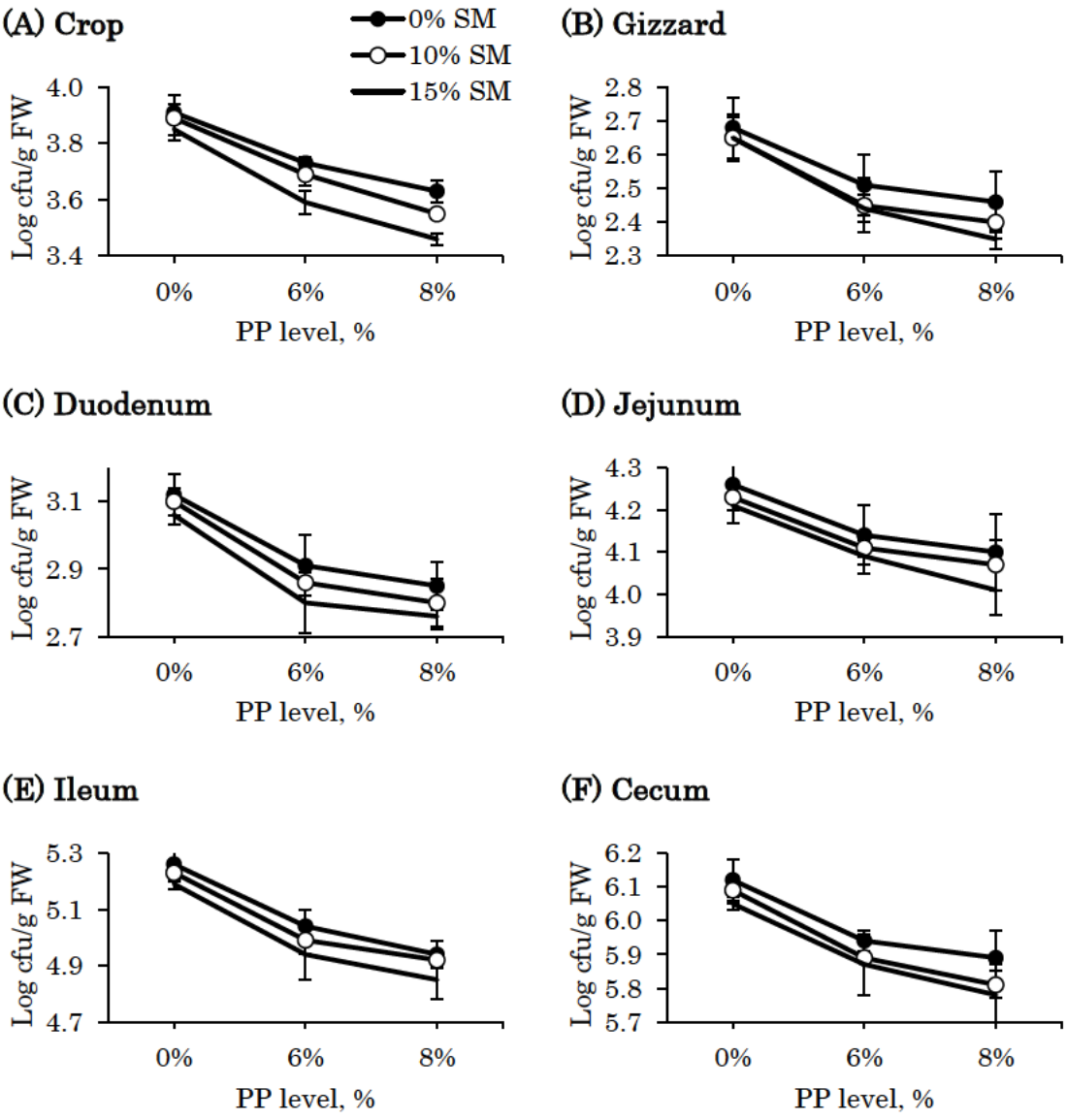
[A-C] Mean values within the row followed by different superscript letters are significantly different at the $P < 0.05$ level.

[a-c] Mean values within the column of PP groups followed by different superscript letters are significantly different at the $P < 0.05$ level.

[1] SM = shrimp meal, PP = persimmon peel.

[2] The values (except for diet) represent mean ± standard error of 12 observations.

[3] One unit (U) of the enzyme activity was defined as the amount of enzyme which produced 1 μmol of *N*-acetylglucosamine per h at 37°C with desired pH values depending on the pH of each part of the GI tract.



Source of variation	Crop	Gizzard	Duodenum	Jejunum	Ileum	Cecum
SM	$P < 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
PP	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
SM × PP	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$

Fig. IV-3. ***Escherichia coli*** **counts in digesta from different parts of the gastrointestinal tract (crop, gizzard, duodenum, jejunum, ileum, and cecum) of laying hens fed with diets containing shrimp meal and persimmon peel.** SM = shrimp meal, PP = persimmon peel, FW = fresh weight. Data represent mean ± standard error (n = 4).

Table IV-4. **Condensed tannin (mg/100 g DM) of the diets, digesta obtained from different parts of the gastrointestinal tract, and excreta in laying hens[1]**

Dietary groups		Diets	The gastrointestinal parts						Excreta
			Crop	Gizzard	Duodenum	Jejunum	Ileum	Cecum	
Time after feeding, min			30	30	60	60	60	60	
0% SM	6% PP	195.9	195.7 ± 4.3^{A}	167.6 ± 6.0^{A}	369.4 ± 10.2^{B}	449.5 ± 12.3^{C}	679.8 ± 20.0^{D}	686.2 ± 15.4^{Da}	728.0 ± 17.6^{D}
	8% PP	262.9	262.9 ± 8.4^{A}	230.2 ± 7.3^{A}	526.1 ± 10.7^{B}	638.4 ± 10.5^{C}	1,022.5 ± 30.3^{D}	1,038.6 ± 27.3^{Db}	1,068.3 ± 30.1^{D}
10% SM	6% PP	196.8	196.4 ± 4.7^{A}	164.4 ± 2.9^{A}	347.4 ± 7.4^{B}	417.6 ± 13.5^{C}	587.3 ± 17.7^{D}	589.6 ± 14.2^{Dc}	623.7 ± 15.9^{D}
	8% PP	263.1	262.6 ± 6.9^{A}	228.9 ± 8.8^{A}	498.7 ± 8.6^{B}	597.1 ± 15.6^{C}	876.8 ± 15.6^{D}	889.2 ± 23.0^{Dd}	928.7 ± 26.5^{D}
15% SM	6% PP	196.2	196.5 ± 3.2^{A}	163.4 ± 2.6^{A}	335.3 ± 9.9^{B}	381.5 ± 10.6^{B}	497.1 ± 15.4^{C}	512.7 ± 15.7^{Cc}	524.7 ± 19.2^{C}
	8% PP	262.3	263.4 ± 6.8^{A}	227.4 ± 4.3^{A}	478.4 ± 12.4^{B}	540.6 ± 13.2^{C}	742.2 ± 17.5^{D}	750.4 ± 14.1^{Da}	765.0 ± 14.1^{D}
Source of variation			---------- *P*-value ----------						
SM			0.9615	0.8174	0.0025	0.0001	0.0001	0.0001	0.0001
PP			0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
SM × PP			0.9547	0.9872	0.8098	0.5027	0.0769	0.0249	0.0860

[A-D] Mean values within the same row followed by different superscript letters are significantly different at the $P < 0.05$ level.

[a-d] Mean values within the same column followed by different superscript letters are significantly different at the $P < 0.05$ level.

[1] SM = shrimp meal, PP = persimmon peel.

[2] The values (except for diet) represent mean ± standard error of 4 observations.

[3] The values (except for diet) represent mean ± standard error of 12 observations

Discussion

Chapter 2 showed that *in vitro* digestibility of SM diets was improved by the addition of PP, and DM digestibility reached maximum when 8% of PP was added, but CP digestibility 6%. Chapter 3, in which egg productivity was studied, suggested that similar phenomena occurred also in the *in vivo*, but CP digestibility seemed not to be deteriorated when 8% of PP was added to SM diets. The present chapter, in which ileal digestibility was measured, clarified that dietary PP improved *in vivo* digestibility of SM diets in laying hens, supporting the hypothesis obtained in chapter 3. This is a very important finding for the practical use of PP as a digestion promoter of SM diets. This improvement may be involved with chitinase in PP, and hence the involvement of dietary PP on chitinase activity in each segment of the GI tract is discussed below.

The chitinase activity in each intestinal content excepting the ileal and cecal contents was enhanced when PP was added to diets, and the magnitude increased with increasing level of PP: the greatest enhancement was found in the crop content. However, the intrinsic chitinase activity in gizzard content was as high as 2,300 U/g of protein (23 times higher than that in crop content), and therefore the enhancement of chitinase activity by dietary PP was not remarkable even when 8% of PP was added. This indicates that dietary PP increased SM digestibility with a limited contribution to total chitinase activity. It is impossible to explain this strange phenomenon, but at least, the feed storage time in the crop, in which little endogenous chitinase activity and high PP chitinase activity were found, may not be the candidate, because the feed storage time in the crop and gizzard were reported to be similar (Van Der Klis *et al*., 1990). Further study is required to understand the details.

Chapter 3 showed that PP reduced ammonia excretion, which suggests the reduction of bad bacteria producing ammonia, such as *E. coli* (Vince *et al*., 1973), by PP, and, in

fact, the present chapter confirmed that *E. coli* count was significantly reduced by PP in each segment of the GI tract. In this connection, SM reduced *E. coli* count only in the crop, suggesting that PP is the main factor of the *E. coli* reduction. Candidate chemicals of *E. coli* reduction in PP would be chitinase and tannin because the former is known as an enzyme for biological defense in plants (Taira *et al*., 2002; Lawrence and Novak, 2006) and animals (Suzuki *et al.,* 2002; Tabata *et al*., 2018): the chitinase present in the stomach of animals is believed to function to suppress bacteria contained in feed. However, as mentioned above, PP chitinase activity was much lower than intrinsic one in the stomach, and PP was effective to reduce *E. coli* also in the ileum and cecum where little chitinase activity was observed. Therefore, PP chitinase may not be the main factor to decrease *E. coli* count. On the other hand, tannin level increased toward the distal part of the digestive tract, probably because of its low digestibility. In this connection, there are several reports showing that tannins effectively reduce the number of intestinal bacteria (Smith and Mackie, 2004; Jamroz *et al*., 2009). Consequently, tannin seems to be a promising factor to decrease *E. coli* count. Unfortunately, the effect of isolated PP tannin on intestinal bacteria in chickens remains unsolved.

Conclusion

This chapter proved that PP increased *in vivo* digestibility of SM, but the contribution of PP chitinase to SM digestion was not clarified, because of much lower chitinase activity in PP compared with intrinsic one, therefore further studies are needed to understand. In addition, it was revealed that PP reduced *E. coli* count in the intestinal tract, and tannin in PP may be the main factor of this reduction. Consequently, it is suggested that dietary PP can potentially serve as a digestion promoter for chitin-rich SM diets and improve the intestinal environment of laying hens.

Chapter 5

General Discussion

The present study revealed that SM utilization could be improved by substituting maize with PP. This method may be superior to the formic method by Rahman and Koh (2016a), because of no acid-treatment process (time- and labor-saving) and no cogeneration of acidic waste solution. However, it may be less suitable to use SM diets containing PP for broilers, because dietary PP cannot reduce high level of calcium (Ca) in SM, and an excessive level of Ca in diets can be an anorectic factor that depresses the growth rate of broilers (Watkins *et al*., 1989; Gautier *et al*., 2017). To prevent excessive Ca intake, a limited amount of SM at 5% can be added for broiler diets (Rahman and Koh, 2016c). Taking these into account, the formic acid method by Rahman and Koh (2016b, 2016c) should be selected for broiler diets, because formic acid treatment could reduce Ca level in SM, and formic acid-treated SM could be included up to 10% in broiler diets. Consequently, it is interesting to investigate the possibility of feeding diets containing formic acid-treated SM along with dietary PP for broilers.

In addition, the present study also revealed the beneficial effects of dietary SM and PP on egg production. For the egg industry worldwide, inclusive of Japan and Thailand, the production of eggs which are of good eggshell quality and egg internal quality is important to the economics of the industry. In the present study, dietary PP was proved to have positive effects on improving albumen quality, such as Haugh unit and albumen height. Consistent with the present findings, Oh *et al*. (2013) also reported an improvement in the Haugh unit values after 7 days of egg storage in laying hens fed with dietary supplementation with PP. Accordingly, use PP as a feed constituent for laying hen diets may lead to the production of value-added eggs.

Yolk color is one of the critical factors for marketing. Generally, the preference for yolk color varies considerably depending on the part of the world, and in Thailand, most consumers prefer dark red color yolks (about 11 color score) (Lokaewmanee *et al*., 2009). According to the yolk-color enhancing effect of SM observed in this study, this may not only satisfy the desired yolk color for purchasers, but also a reduction in use of synthetic pigmentation substances, which are expensive and not approved for use in many countries, including Japan and Thailand.

Additionally, the present findings also revealed that the antioxidant capacity of yolks could be improved by dietary supplementation with both SM and PP, and this enhancing effect can contribute to the production of antioxidant-enriched eggs. As is well known, an intake of antioxidants through diets can reduce oxidative damage in cells and improve human health (Erba *et al*., 2005; Maffei *et al*., 2007). Taking these into account, consumption of antioxidant-enriched eggs may promote consumer health, and this medicinal property may probably enlarge economic value for egg production.

Eggshell quality is another factor that directly impacts farmer income. A problem with poor eggshell quality will result in a downgrade of eggs, as well as economic loss to egg producers. In the present study, the finding found that eggs laid by hens fed with SM-supplemented diets had a significant improvement in the quality of eggshells, including eggshell strength, weight, and thickness. This improvement would have a significant economic impact on the egg production industry, particularly in prolonging the economic life of aged laying hens, because shell quality deteriorated when laying hens get older (Roland, 1980). Additionally, such improvements may contribute to a decrease in the risk of extraneous bacteria penetration into eggs through a cracked eggshell surface, causing spoiled eggs, and also a reduction in using $CaCO_3$, an expensive inorganic mineral source, in the poultry industry.

The improvement of nutritional utilization of SM by PP can lead to not only promote the consumption of SM but also the reduction of using expensive protein sources, such as soybean meal. Comparing with the imported conventional protein source like soybean meal (approximately 386.2 USD/ton), SM (approximately 100-120 USD/ton) is much cheaper and locally available in shrimp-producing countries, such as Thailand and other Southeast Asian countries. Consequently, use SM as a feed alternative constituent for poultry production considerably reduces feed costs.

Unfortunately, persimmon production is very small in Thailand, and hence PP products should be imported from foreign countries if PP is used for poultry feed in this country. This does not meet the policy of "Local Production for Local Consumption". Consequently, alternatives should be found if SM is used practically in Thailand. In this connection, pepper and its by-products may be a good candidate, because chitinase has been reported to exist in pepper fruits (Long *et al*., 2018), and there is the expression of chitinase mRNA in several parts of pepper, such as floral organs, leaves, and root endodermis (Hong *et al*., 2000; Hong and Hwang, 2002). Pepper is widely cultivated in Southeast Asia countries, especially in Thailand, and thus, due to a large amount of pepper production, it is so easy to obtain a large amount of its by-products, such as stalks, stems, and leaves. Taking these into account, it is interesting to investigate a novel chitinase source in pepper and its by-products and use them as alternatives to PP for poultry production in Thailand.

In recent years, insect feed has been receiving attention as a substitute for fish meal, which has reduced in supply due to the depletion of marine resources. However, high chitin level in insect meal is a great concern for use of this by-product for animal diets. Therefore, PP may be effective in improving some insect feed. Besides, PP may be suitable as an ingredient in the fish feed, because shrimp and crab shells are commonly used in the fish feed. Given that although chitinolytic activity exists in the digestive tract

of fish (Gutowska *et al*., 2004; Fines and Holt, 2010), the activity was too low to use dietary chitin as the main ingredient source (Shiau and Yu, 1999).

Nowadays, global warming is a great concern for livestock farmers, given that hot weather can harmfully affect animal productivity. In poultry, heat-stress induced by hot weather can lead to a decrease in productivity, such as BW, FI, egg production, and egg quality (Muiruri and Harrison, 1991; Mahmoud *et al*., 1996; Mashaly *et al*., 2004), including depresses in immune functions, causing an increase in mortality rate (Mashaly *et al*., 2004). According to Tuzcu *et al*. (2008) and Dong *et al*. (2015), polyphenols can alleviate oxidative stress induced by hot weather and recover the productivity of poultry. In this connection, persimmon fruits are known to be a rich source of powerful antioxidants, such as carotenoids, polyphenols, and tannins (Takahashi *et al*., 2006; Gu *et al*., 2008). Besides, in the present study, an enhance in the antioxidant capacity of egg yolks in hens fed with PP-supplemented diets was observed, which is a sign of a hens' enhanced antioxidant status. Thus, by this finding, PP may potentially serve as a feed constituent to promote the heat-resistance in chickens, but such effects of PP were not practically examined in this study, and hence it is worth investigating, especially in tropical countries.

Concluding Remarks

SM, a by-product of shrimp-processing industries having high level of protein, has rarely been used as an alternative protein source for poultry feed, because of being difficult to be digested due to its high chitin level. To improve the nutrient quality and feeding values of this by-product, this research had been designed to use PP, a by-product of dried persimmon processing having high chitinase activity, as a digestion promoting constituent for SM diets for laying hens. The obtained results clarified that dietary PP could improve the feeding value of SM and has advantageous effects on laying hens fed with SM-supplemented diets, including promoting digestibility, recovering the deteriorated laying performance induced by the negative nutritional effect of SM, improving the quality of eggs, and reducing unfavorable intestinal bacteria. These show that by combining shrimp by-product meal and dried persimmon by-product, it is possible to create a new protein feed for laying hens with functionality. It possibly contributes to promoting self-sufficient feed production by utilizing local resources.

For Future Research

From the practical viewpoint, further studies are needed to investigate whether dietary containing formic acid-treated SM and PP can be used for broilers. In addition, polyphenols rich in PP have been reported an efficiency on alleviate oxidative stress induced by hot weather and recover productivity in poultry, but such effects of PP have not been evaluated in this study, and thus it is worth investigating.

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