Animal Science Journal
Animal Science Journal (2012) **83,** 594–598 doi: 10.1111/j.1740-0929.2011.00999.x

ORIGINAL ARTICLE

Feeding *Aspergillus awamori* reduces skeletal muscle protein breakdown and stimulates growth in broilers

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ABSTRACT

This study was conducted to show that dietary supplementation of a fungus, *Aspergillus awamori* called Koji in Japan, reduces skeletal muscle protein breakdown and stimulates growth in broiler chickens. A total of 30 chicks at 15 days of age was divided into control and two treatment groups (10 birds per treatment). Control group was fed basal diet and treatment groups were fed the basal diets supplemented with *A. awamori* at levels of 0.05% and 0.2%. The birds were raised for 12 days from 15 to 27 days of age and then the effect on growth, organ weights and plasma 3-methylhistidine concentration and digestibilities of protein and energy was evaluated. The messenger RNAs (mRNAs) of atrogin-1, ubiquitin, proteasome, m-calpain, μ-calpain, β-actin, myosin and pax-7 in the breast muscle were also measured. Body weight gain and breast muscle weight were increased, although feed intake was decreased by the fungus and thus feed efficiency was increased. Protein and energy digestibilities were increased. Furthermore, plasma 3-methylhistidine concentration was decreased by the fungus. The mRNAs of atrogin-1, ubiquitin, proteasome, m-calpain and μ-calpain were all decreased. The mRNA of β-actin but not myosin and pax-7 was slightly increased by the fungus. In conclusion, feeding *A. awamori* improves growth performance because skeletal muscle proteolytic activity is reduced and digestibilities of energy and protein are increased.

Key words: *Aspergillus awamori, broilers, fungus, growth, probiotics.*

INTRODUCTION

Aspergillus awamori is a fungus called 'Koji' in Japan and has long been used for food processing. The products processed by *A. awamori* are given GRAS (Generally Recognized as Safe) status from the Food and Drug Administration (Bigelis & Lasure 1987). *A. awamori* is used for processing Japanese food such as Shochu, a traditional Japanese liquor. As it has been reported that distillery by-product of Shochu contains an unidentified growth factor for broiler chickens (Mahfudz *et al.* 1996a,b, 1997), it is proven that *A. awamori* produces a growth promoter during fermentation. In addition, Koji is well known to produce enzymes enhancing digestion of carbohydrates and proteins (Gracia *et al.* 2003). Thus, improving growth performance by feeding *A. awamori* is probable. In fact, Yamamoto *et al.* (2007) have reported that a fermented product using *A. awamori* (Koji-feed) promotes growth in broiler chickens. Also, Kamizono *et al.* (2010) have reported that shochu distillery by-product decreases gene expression of enzymes responsible for regulating skeletal muscle protein metabolism and stimulates growth in broiler chickens. From these results, we thought that *A. awamori* might stimulate growth in broiler chickens. To confirm this assumption, we conducted an experiment to examine the effect of *A. awamori* on growth performance, digestibility and skeletal muscle protein metabolism in broilers. Many antibiotics have been used to promote animal health and to improve utilization of nutrients and growth performance in poultry (Babalola *et al.* 2006). However, there is strong public and governmental concern about the increasing prevalence of resistance to antibiotics in disease-causing bacteria because many antibiotics now available to treat human diseases will become ineffective in the future (Casewell *et al.* 2003). So replacing antibiotics

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Received 27 July 2011; accepted for publication 13 October 2011.

with probiotics is very important. Thus, the additional purpose of the present study was to evaluate *A. awamori* as a probiotic.

MATERIALS AND METHODS

Animals and feed

The animal experiment was conducted in accordance with the guidelines of Kagoshima University. Thirty one-day-old male broiler chickens (Chunky strain) were supplied by a commercial hatchery (Kumiai Hina Center, Kagoshima, Japan). Chicks were housed in an electrically heated battery brooder, and provided with water and commercial starter diet (23% crude protein (CP) and 3081 kcal/kg metabolizable energy(ME)) (Nichiwa Sangyou Company Kagoshima, Japan) until 12 days of age. Then chicks were fed the basal diet from 12 days of age to 15 days of age. The composition of the basal diet (CP 22. 6%, ME 3081 kcal/kg) is shown in Table 1. Chicks were divided into three groups ($n = 10$): control and *A. awamori* groups with two levels of koji-mold (0.05% and 0.2%). Koji-mold in *A. awamori* was made from rice in the conventional manner. The koji was mixed in the basal diet. The numbers of *A. awamori* spores given were about 25×10^4 and 10×10^5/g feed for diets of 0.05 and 0. 2% groups, respectively. The birds were given the experimental diets from 15 to 27 days of age. *A. awamori* was prepared at Gen koji Research Institute (Kagoshima, Japan). The experiment was conducted in a temperature-controlled room with 14 h light and 10 h dark cycle. Room temperature was kept at 25°C with relative humidity 50–70% throughout the experiment.

Parameters measured

Body weight was recorded every 3 days and feed intake was recorded daily during the experimental period. At the end of the experimental period, the birds were slaughtered and then dissected to measure the weights of breast muscles, liver and abdominal fat. Blood samples were collected into heparinized test tubes, quickly centrifuged at $5900 \times g$ for 10 min at 4°C to separate plasma, and stored at −30°C until analysis.

The plasma 3-methylhistidine concentration was measured by high performance liquid chromatography (HPLC) method according to Hayashi *et al.* (1987). Protein was measured by a macro-corder machine (J-Science Lab Co., Ltd, Kyoto, Japan) and energy by a bomb calorie meter (Yoshida, Tokyo, Japan). Total RNA was extracted from a piece of pectoralis superficial muscle (about 100 mg) using an RNeasy® Fibrous Tissue Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The RNA concentration and purity were determined spectrophotometrically using A260 and A280 values in a photometer (BioPhotometer, Eppendorf, Hamburg, Germany). The ratio of A260/A280 for all samples was between 1.8 and 2.0. Complementary DNA (cDNA) was synthesized at 800 ng RNA per 20 μL of reaction solution with PrimeScript® RT reagent Kit (Perfect Real Time, Takara, Shiga, Japan) by the Program Temp Control System PC320 (Astec, Fukuoka, Japan), which was set at reverse transcription 37°C for 15 min, inactivation of reverse transcriptase at 85°C for 5 s, and refrigeration at 4°C for 5 min. Real-time PCR primers were prepared as previously described. Gene expression was measured by real-time PCR using the 7300 Real Time PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR® Premix Ex TaqTM (Perfect Real Time, Takara). The thermal cycle was as follows: 1 cycle at 95°C for 10 s, and 60 cycles at 95°C for 5 s and 60°C for 31 s. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) was used as an internal standard and was not significantly different among the two experimental groups. Gene expression results are shown as percentage of the control value.

Table 1 Composition and nutrient analysis of basal diet

Ingredients,%	Diet
Corn	50.19
Alfalfa meal	2.64
Soybean meal	39.01
Corn oil	4.40
L-Lysine HC1	0.01
DL-methionine	0.18
Mineral† mix	3.31
Vitamin† mix	0.26
Calculated analysis	
Crude protein, %	22.60
Metabolizable energy, kcal/kg	3081
Ca, %	1.10
P, %	0.46
Na, %	0.26
Cl, %	0.25

†The mineral-vitamin permix supplied per kilogram of feed: 154 mg of Mn, 121 mg of Zn, 176 mg of Fe, 33 mg of Cu, 1.1 mg of I, 0.7 mg of Se, 11.000 IU of vitamin A, 2.640 IU of vitamin D, 121 IU of vitamin E,12 mg of vitamin B_{12}, 1.37 mg of retinol, 0.13 mg of cholecalciferol, 6.50 mg of riboflavin, 2.60 mg of thiamine hydrochloride, 1.30 mg of pyridoxamine hydrochloride, 0.03 mg of cyanocobalamin, 10.40 mg of D-pantothenic acid, 26.00 mg of nicotinic acid, 1.05 mg of vitamin K3, 0.52 mg of pteroylglutamic acid, 0.78 mg of choline chloride, 0.07 mg of biotin, 2.54 g of sucrose.

Statistical analysis

The differences among treatment groups and the control group were analyzed by by GLM using SPSS Statistics 17.0 2008 (SPSS Inc., Chicago, IL, US). The significant differences among means of treatments were compared by Duncan's new multiple-range test. $P \leq 0.05$ was set as limit of significance.

RESULTS

Effects of *A. awamori* on body weight gain, feed intake, feed conversion ratio, breast muscle weight, digestibilities of protein and energy and plasma 3-methylhistidine concentration are summarized in Table 2. *A. awamori* increased body weight gain significantly ($P < 0.05$) when the level was 0.05% but not at the level of 0.2%. Feed intake was decreased ($P < 0.01$) in the 0.05% *A. awamori* group but not in the 0.2% group. Thus, feed conversion ratio was significantly decreased in the 0.05% group. The breast muscle weights were significantly increased ($P < 0.01$) in both treatment groups. Protein and energy digestibilities were also significantly increased in both treatment groups. Plasma 3-MH was decreased significantly by *A. awamori* at both feeding levels.

Table 2 Effect of dietary *Aspergillus awamori* on body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), breast muscle weight (BMW), protein and energy digestibilities and plasma 3-MH

	Control	*A. awamori*	
		0.05%	0.2%
Initial BW(g)	399 ± 9	399 ± 9	399 ± 9
BWG (g/12 day)	691 ± 40[b]	808 ± 15[a]	704 ± 33[b]
FI (g/12 day)	1197 ± 36[a]	1057 ± 37[b]	1098 ± 26[ab]
FCR	1.7 ± 0.14[a]	1.3 ± 0.04[b]	1.5 ± 0.05[ab]
BMW (g/100g BW)	23.9 ± 1.1[b]	28.8 ± 0.4[a]	27.3 ± 1[a]
Protein Digestibility (%)	59 ± 4.6[b]	75 ± 4.1[a]	76 ± 4.4[a]
Energy digestibility (%)	64 ± 4[b]	74 ± 3[a]	73 ± 3[a]
Plasma 3-MH (μ mol/ mL)	30.2 ± 1.5[a]	15.8 ± 1.1[b]	18.7 ± 1.3[ab]

Values are expressed as means ± SD; [a–c]Means with different superscripts differ from each other significantly.

Figure 1 shows the effect of *A. awamori* on mRNA concentrations of atrogin-1, ubiquitin, proteasome, m-calpain and μ-calpain. The mRNAs of atrogin-1, ubiquitin, proteasome, m-calpain and μ-calpain were all decreased by the fungus. However, the effects on atrogin-1 and m-calpain mRNAs were not statistically significant. Figure 2 shows the effect of the *A. awamori* on mRNA concentrations of β-actin, myosin and pax-7. The mRNA of β-actin was increased by the fungus. However, mRNAs of pax-7 and myosin were not affected by *A. awamori*.

DISCUSSION

The major aim of the present study was to show growth stimulation by *A. awamori* feeding and its mechanism in broiler chickens. The improvement in weight gain and feed efficiency due to *Aspergillus* may be partially due to the increase in ME of the feed. Birds do not produce enzymes such as cellulase and xylanase which are required for the digestion of soluble non-starch polysaccharides (NSPs). These enzymes can be produced by *Aspergillus* and thus digestibility might be improved by feeding *A. awamori* in the present study. Both protein and energy digestibilities were significantly increased by the fungus in the present experiment. This is consistent with the results of Yamamoto *et al*. (2005) indicating that Koji-feed increases digestibility in broilers. Enzymes contained in Koji might stimulate digestion and improve growth. Saleh *et al*. (2005) have reported that exogenous enzymes had potential to improve broiler performance. Furthermore, Amsal *et al*. (1999) found that *A. awamori* possesses the ability to digest raw starches. These may be the reason for the efficient feed utilization due to *Aspergillus* addition. It is also probable that *Aspergillus* could improve nutritional quality of soybean meal since trypsin inhibitor contained in soybean is degraded by *Aspergillus* (Hong *et al*. 2004).

Breast muscle weight was increased ($P < 0.05$) by the fungus. Yamamoto *et al*. (2007) also reported that when broilers were fed on diets containing 0.05% and

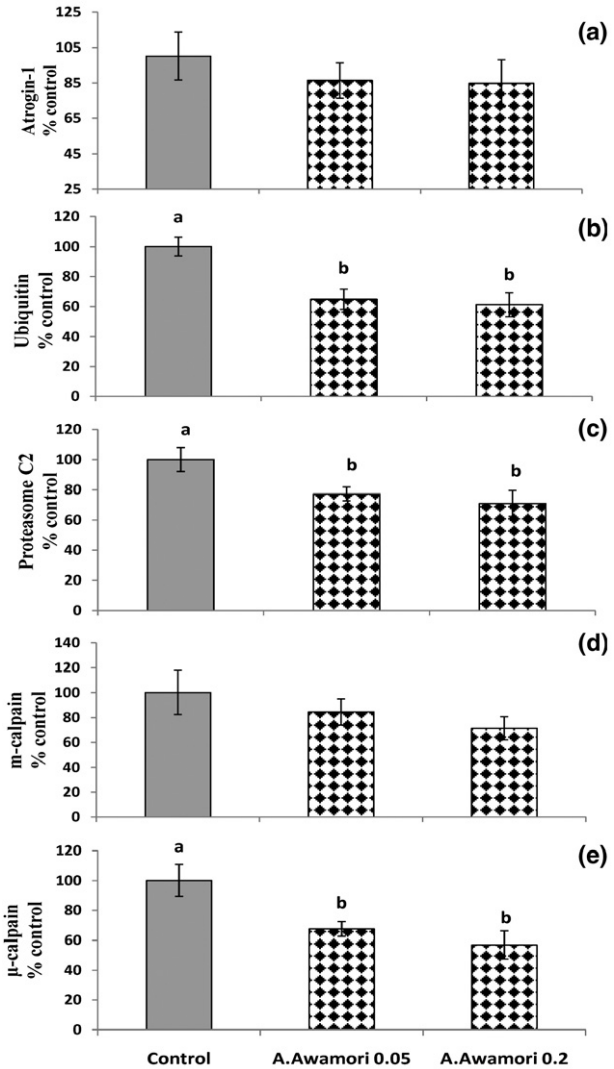

Figure 1 Effect of dietary *Aspergillus awamori* (0.05, 0.2%) on mRNAs of atrogen-1 (A), ubiquitin (B), proteasome (C), m-calpain (D) and μ-calpain (E) contents in muscle. Values are expressed as percentage of the control values (means ± SD); [a–c]Means with different superscripts differ from each other ($P < 0.05$).

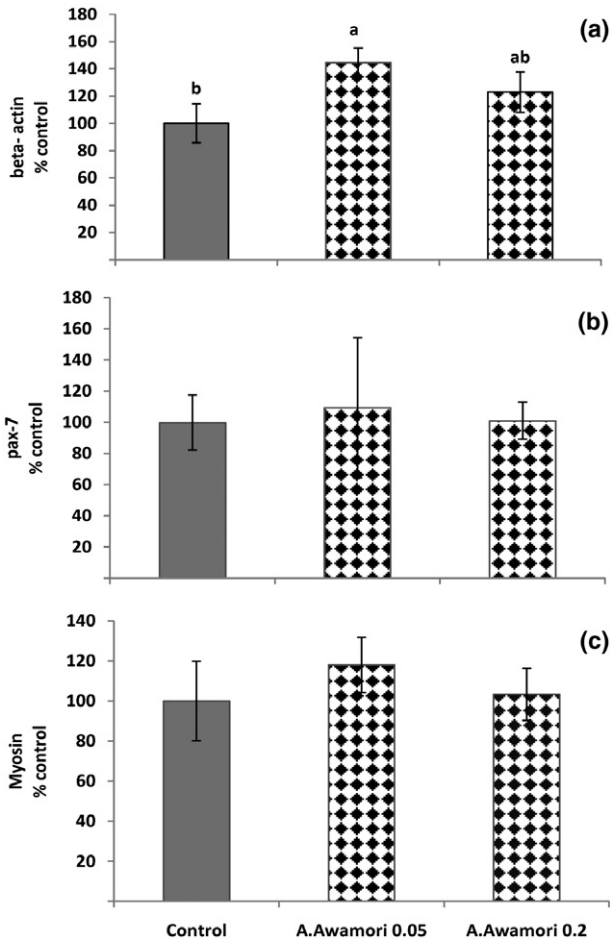

Figure 2 Effect of dietary *Aspergillus awamori* (0.05, 0.2%) on mRNAs of β-actin (A), pax-7 (B) and myosin (C) contents in muscle. Values are expressed as percentage of the control values (means ± SD); [a–c]Means with different superscripts differ from each other ($P < 0.05$).

1% of Koji-feed, carcass weight was significantly increased and the breast muscle weight tended to increase. This seems to be due to a growth promoter reported by Kamizono *et al*. (2010). The growth promoting effect of the fungus can be explained by the effect on plasma 3-methylhistidine concentration. The plasma 3-methylhisitidine concentration was decreased by the fungus, indicating a decreased rate of skeletal muscle protein degradation. Measurement of urinary 3-methylhisitidine excretion is widely used as an index of muscle protein degradation, but it is difficult to measure 3-methylhistidine excretion in chickens. Thus, we used plasma 3-methylhisitidine concentration to track changes in muscle protein degradation as reported by Nagasawa *et al*. (1998).

Muscle mRNAs of atrogin-1, ubiquitin, proteasome, m-calpain and μ-calpain play roles in the control of degradation of sarcoplasmic proteins. mRNAs of ubiquitin, proteasome and μ-calpain were significantly decreased by the fungus and those of atrogin-1 and m-calpain tended to decrease (Fig. 1). These results support the idea that muscle growth promotion due to *A. awamori* is caused by suppression of skeletal muscle protein degradation. The present results are consistent with the results of Kamizono *et al*. (2010), indicating that Shochu distillery by-product reduces gene expressions of enzymes responsible for skeletal muscle protein breakdown. Proteasome is thought to play a major role in the degradation of most sarcoplasmic proteins, and calpain might play a significant role in initiating muscle protein degradation by releasing protein fragments for proteolysis of the ubiquitin–proteasome system. Atrogin-1 was identified as a muscle-specific E3 that was highly expressed in muscle atrophy (Bodine *et al*. 2001; Gomes *et al*. 2001). Calpains are a family of intracellular Ca^{2+}-dependent cysteine proteases that are ubiquitously expressed in many cells and tissues (Suzuki *et al*. 1995). Two major forms of calpains (m-calpain and μ-calpain) are activated in the presence of micromolar and millimolar Ca^{2+}, respectively (Goll *et al*. 2003). They are heterodimers composed of a large catalytic subunit (80 kDa) and a small regulatory subunit (30 kDa). Smith and Dodd (2007) have reported that calpain acts upstream of the ubiquitin-proteasome system and m-calpain mRNA is scarcely expressed in the muscle and liver in chicken. Expressions of mRNAs of atrogin-1, ubiquitin, and μ-calpain large subunit were significantly decreased by *A. awamori* (Fig. 1), indicating that *A. awamori* decreased muscle protein breakdown. The mRNAs of β-actin, myosin and pax-7 were all increased but only slightly by the fungus. These genes are related to growth and development of muscle fiber (Somi *et al*. 2006; Guobin *et al*. 2011). These results support our idea that decreased skeletal muscle protein breakdown is responsible for the skeletal muscle growth stimulation due to *A. awamori* feeding.

Conclusion

In conclusion, this study shows that feeding *A. awamori* improves growth performance mainly due to its effect on skeletal muscle protein breakdown and could be used as an effective probiotic in broilers.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (no. 19208024). We are grateful to Kagoshima Chicken Foods Co., Ltd (Kagoshima, Japan) for a supply of broiler chicks.

REFERENCES

Amsal A, Takigami M, Ito H. 1999. Increased digestibility of raw starches by mutant strains of *Aspergillus Awamori*. *Food Science and Technology Research* **5,** 153–155.

Babalola TOO, Apata DF, Atteh JO. 2006. Effect of β-xylanase supplementation of boiled castor seed meal-based diets on the performance, nutrient absorbability and some blood constituents of pullet chicks. *Tropical Science* **46,** 216–223.

Bigelis R, Lasure LL. 1987. Fungal enzymes and primary metabolites used in food processing. In: Beuchat LR (ed.), *Food and Beverage Mycology*, 2nd edn., pp. 473–516. Avi, Westport, CT.

Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, Glass DJ. 2001. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* **294,** 1704–1708.

Casewell M, Friis C, Marco E, McMullin P, Phillips I. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *Journal of Antimicrobial Chemotherapy* **52,** 159–161.

Goll DE, Thompson VF, Li H, Wei W, Cong J. 2003. The Calpain System. *Physiological Reviews* **83,** 731–801.

Gomes MD, Lecker SH, Jagoe RT, Navon A, Goldberg AL. 2001. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 14440–14445.

Gracia MI, Aranibar MJ, Lazaro R, Medel P, Mateos GG. 2003. A-Amylase supplementation of broiler diets based on corn. *Poultry Science* **82,** 436–442.

Guobin C, Xiangping L, Jing L, Rong C, Deqin L, Ying Z, Aiqing D, Teng M, Wei Z, Kehua W, Guohong C. 2011. Temporal and Spatial Expression of the Pax-7 Gene During Chicken Embryo and Postnatal Development. *Journal of Animal and Veterinary Advances* **10,** 1785–1788.

Hayashi K, Maeda Y, Toyomizu M, Tomita Y. 1987. High-performance liquid chromatographic method for the analysis of N^{τ}-methylhistidine in food, chicken excreta, and rat urine. *Journal of Nutritional Science and Vitaminology* **33,** 151–156.

Hong KL, Lee CH, Kim SW. 2004. *Aspergillus oryzae* GB-107 fermentation improves nutritional quality of food soybeans and feed soybean meals. *Journal of Medicinal Food* **7,** 430–435.

Kamizono T, Nakashima K, Ohtsuka A, Hayashi K. 2010. Effects of feeding hexane- extracts of a shochu distillery by-product on skeletal muscle protein degradation in broiler chicken. *Bioscience, Biotechnology, Biochemistry* **74,** 92–95.

Mahfudz LD, Hayashi K, Ikeda M, Hamada K, Ohtsuka A, Tomita Y. 1996a. The effective use of shochu distillery by-product as a source of broiler feed. *Japanese Poultry Science* **33,** 1–7.

Mahfudz LD, Hayashi K, Otsuji Y, Ohtsuka A, Tomita Y. 1996b. Separation of growth promoting factor of broiler chicken from shochu distillery by-product. *Japanese Poultry Science* **33,** 96–103.

Mahfudz LD, Nakashima K, Ohtsuka A, Hayashi K. 1997. Growth factors for a primary chick muscle cell culture from shochu distillery by-product. *Bioscience, Biotechnology, Biochemistry* **11,** 1844–1847.

Nagasawa T, Hirano J, Yoshizawa F, Nishizawa N. 1998. Myofibrillar protein catabolism is rapidly suppressed following protein feeding. *Bioscience, Biotechnology, Biochemistry* **62,** 1932–1937.

Saleh F, Tahir M, Ohtsuka A, Hayashi K. 2005. A mixture of pure cellulase, hemicellulase and pectinase improves broiler performance. *British Journal of Poultry Science* **64,** 602–606.

Smith IJ, Dodd SL. 2007. Calpain activation causes a proteasome-dependent increase in protein degradation and inhibits the Akt signalling pathway in rat diaphragm muscle. *The Journal of Physiology* **92,** 561–573.

Somi S, Klein ATJ, Houweling AC, Ruijter JM, Buffing AAM, Moorman AFM, van den Hoff MJB. 2006. Atrial and ventricular myosin heavy-chain expression in the developing chicken heart: strengths and limitations of non-radioactive in situ hybridization. *Journal of Histochemistry & Cytochemistry* **54,** 649–664.

Suzuki K, Sorimachi H, Yoshizawa T, Kinbara K, Ishiura S. 1995. Calpain: novel family members, activation, and physiologic function. *Biological Chemistry Hoppe-Seyler* **376,** 523–529.

Yamamoto M, Saleh F, Ohtsuka A, Hayashi K. 2005. New fermentation technique to process fish waste. *Animal Science Journal* **76,** 245–248.

Yamamoto M, Saleh F, Tahir M, Ohtsuka A, Hayashi K. 2007. The effect of Koji-fed (fermented distillery by-product) on the growth performance and nutrient metabolizability in broiler. *Journal of Poultry Science* **44,** 291–296.