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*Aspergillus Niger r*educes skeletal muscle protein breakdown and stimulates growth in broilers

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Abstract

This study was conducted to show that the inclusion of a fungus, *Aspergillus Niger*, as a dietary supplement in feed reduces protein breakdown in skeletal muscle and stimulates growth in broiler chickens. A total of 24 chicks at 15 d of age were divided into a control group and 3 treatment groups (6 birds per treatment). The control group was fed a basic diet, and the 3 treatment groups were fed the basic diet supplemented with *A. Niger* at a concentration of 0.01, 0.05 and 0.1% respectively. The birds were raised for 12 d from 15 d of age and were evaluated for the fungi's effects on growth, organ weight and plasma 3-methylhistidine concentration as an index of skeletal muscle protein breakdown The mRNAs of atrogin-1, ubiquitin, proteasome and m-calpain were also measured to identify the mechanism underlying the decrement of the muscle protein breakdown resulting from *Aspergillus*. Body weight gain and breast muscle weight were increased even though feed intake by the chicks was decreased by the presence of the fungus, thus improving feed efficiency. Furthermore, plasma 3-methylhistidine concentration was decreased by the fungus. The mRNAs of atrogin-1, ubiquitin, proteasome and m-calpain were decreased, supporting the conclusion that the fungus decreases skeletal muscle protein breakdown. In conclusion, feeding *A. Niger* improves growth performance because proteolytic activity in skeletal muscle is reduced.

Keywords: Aspergillus Niger, Probiotic, Growth, Broiler Chickens

Introduction

Probiotics are defined as live microorganisms which beneficially affect the host animal by improving its intestinal microbial balance. *A. Niger* is used for processing Japanese food such as Shochu, traditional Japanese liquor. Since the distilled by-product of Shochu has been reported to contain an unidentified growth factor for broiler chickens (Mahfudz et al., 1996 a&b, 1997), *A. Niger* was hypothesized to produce a growth promoter during fermentation. In addition, *Aspergillus* produces enzymes that enhance the digestion of carbohydrates and proteins (Gracia et al., 2003), suggesting that growth performance can be improved by feeding *A. awamori*. In the present study, we investigated the effects of *A. Niger* on growth performance and the rate of skeletal muscle protein breakdown in broiler chickens. Thus, an additional purpose of the present study was to evaluate *A. Niger* as a probiotic in broilers diet.

Materials and Methods

Twenty four one-day- old male broiler chicks (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% CP and 3,081 kcal/kg ME, Nichiwa Sangyou Company Kagoshima, Japan) until 12 d of age. Then chicks were fed the basal diet from 12 d of age to 15d of age. The composition of basal diet was (CP 22. 6%, ME 3,081 kcal/kg). Chicks were divided into 4 groups (n= 6): control and *A. Niger* groups with 3 levels of fungus (0.01%, 0.05%, and 0.1%). The fungus was mixed in the basal diet. The birds were given the experimental diets from 15 to 27 days of age. The experiment was conducted in a temperature-controlled room with 14h light: 10 hours dark cycle. Room temperature was kept at 25°C with relative humidity from 50 to 70 % throughout the experiment. Body weight was recorded every 6 days and feed intake.

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 HCL	0.01
DL-Methionine	0.18
Mineral[1] mix	3.31
Vitamin[1] mix	0.26
Calculated analysis	
CP, %	22.60
ME, kcal/kg	3,081
Ca, %	1.10
P, %	0.46
Na, %	0.26
Cl, %	0.25

[1] The mineral- vitamin premix supplied per kilogram of feed: 154mg of Mn, 121mg of Zn, 176mg of Fe, 33mg of Cu,1.1mg of I,0.7mg 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.

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 muscle. Blood samples were collected into heparinised test tubes, to measure plasma 3-methylhistidine concentration. The plasma 3-methylhistidine concentration was measured by HPLC method according to Hayashi et al. (1987). Total RNA was extracted from a piece of pectoralis superficial muscle (about 100mg) using an RNeasy ® Fibrous Tissue Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer s protocol. The RNA concentration and purity were determined using A260 and A280 values measured by a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany). The ratio of A260/A280 for all samples was between 1.8 and 2.0. cDNA was synthesized at 800 ng RNA per 20 ml of reaction solution with the PrimeScript®RT reagent Kit (Perfect Real Time, Takara, Shiga, Japan) by the Program Temp Control System PC320 (Astec, Fukuoka, Japan) using the fllowing protocol:, reverse transcription at 37°C for 15 minutes, inactivation of reverse transcriptase at 85°C for 5 s, and refrigeration at 4°C for 5 minutes. 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, USA) with SYBR®Premix Ex Taq™ (Perfect Real Time, Takara, Shiga, Japan). The thermal cycle protocol was as follows: 1 cycle at 95°C for 10 s, 60 cycles at 95°C for 5 s and 60°C for 31 s. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal standard and did not significantly differ between the two experimental groups. Gene expression results are shown as a percentage of the control value.

Statistical Analysis

The differences among the treatment and control groups were analyzed by the general linear model using SPSS Statistics 17.0 (Statistical Packages for the Social Sciences, released 23 August 2008). The significant differences between the means of treatments were compared by Duncan's new multiple-range test. The limit of significance was established as $P \leq 0.05$.

Results and Discussion

Effects of *A. Niger* on growth performance and plasma 3-methylhistidine concentration are summarized in Table 2. *A. Niger* increased the body weight gain significantly ($P<0.05$) when the level was 0. 01%. Feed intake was decreased ($P<0.01$) in all treatment groups. Feed conversion ratio was significantly decreased in the treatment groups. The muscle weight was significantly increased ($P<0.01$) by *A. Niger*. Plasma 3-methylhistidine concentrations as an index of skeletal muscle protein degradation were lower in the treatment groups, indicating a decreased rate of skeletal muscle protein degradation. Enzymes produced by *Aspergillus* might improve digestibility. It is reported that an enzyme product contained activities of cellulase, hemicellulase, protease, α-amilase and α-galactosidase improves digestibility (Hajati, 2010). These may be the reason for the efficient feed utilization due to *Aspergillus* feeding. Kamizono et al. (2010) have reported the growth promoting effect of Shochu distillery by-product. Shochu is made from many kinds of grains using *A. awamori* for sacharification. Yamamoto et al. (2007) noted that when broilers were fed on diets containing 0.05 and 1% of Koji-feed, carcass weight was significantly increased and the breast muscle weight tended to be increased and abdominal fat was decreased. This seems to be due to the growth promoter produced by *A. Niger*.

Fig. 1 shows that the effect of *A. Niger* on mRNAs concentrations of atrogin-1, ubiquitin, proteasome and m-calpain. The mRNAs of atrogin-1, ubiquitin, proteasome and m-calpain were all decreased by the fungus, but the effects on the levels of the atrogin1 mRNA was not statistically significant. These results

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

	Control	*Aspergillus Niger*		
		0.01%	0.05%	0.10%
BWG, g/12days	635 ± 63^{c}	820 ± 46^{a}	715 ± 7^{b}	730 ± 18^{b}
FI, g/12days	1235 ± 32^{a}	1115 ± 69^{b}	1070 ± 41^{c}	1160 ± 32^{b}
FCR	1.87 ± 0.01^{a}	1.52 ± 0.02^{c}	$1.54 \pm .01^{c}$	1.63 ± 0.05^{b}
BMW, g/100g BW	27.5 ± 0.9^{b}	29.3 ± 1.5^{a}	29.1 ± 0.3^{a}	28.2 ± 0.8^{a}
Plasma 3-MH, µmol/mL	35 ± 4.2^{a}	20 ± 1.2^{b}	18 ± 1.1^{b}	17 ± 1.3^{b}

Values are expressed as mean ± standard error; [a-c] Means different superscripts differ from each other significantly

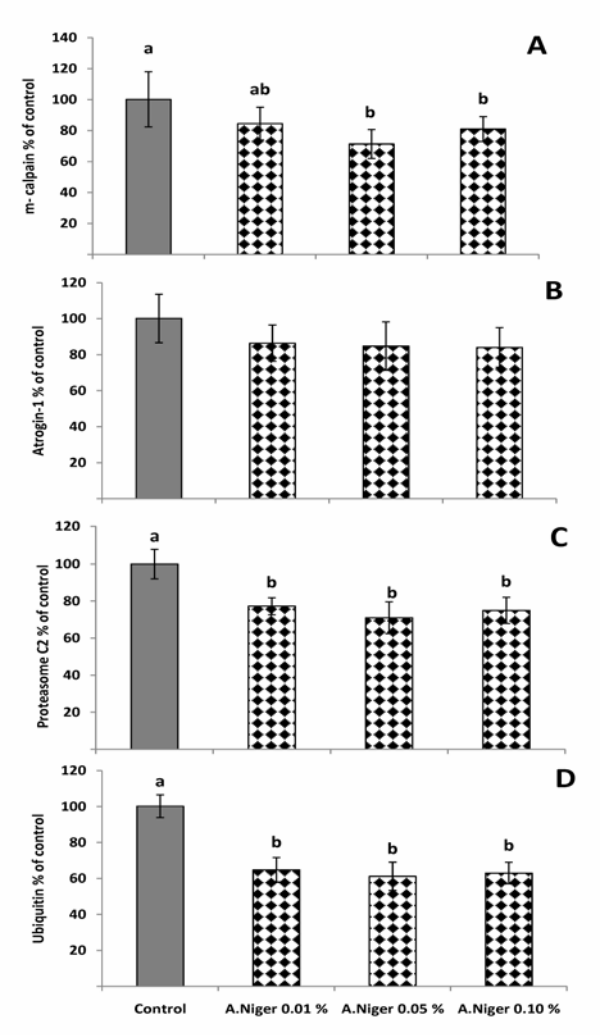

Fig. 1: Effect of dietary *Aspergillus Niger* (0.01, 0.05, 0.1%) on mRNAs of atrogin-1 (A), ubiquitin (B), proteasome (C) and m-calpain (D) contents in muscle. Values are expressed as % of the control values (means± S.D); [a-c] Means with different superscripts differ from each other (P < 0·05)

suggest that the promotion of muscle growth caused by *Aspergillus* is mediated by the suppression of skeletal muscle protein degradation. The proteasome plays a major role in the degradation of most sarcoplasmic proteins, and calpain plays a significant role in initiating muscle protein degradation by releasing protein fragments for proteolysis by the ubiquitin-proteasome system. The ubiquitin-proteasome system is an ATP-dependent proteolysis complex that requires polyubiquitination of the target protein as a marker of degradation by 26S proteasome (Ciechanover, 2006). Polyubiquitination of the target protein consists of the covalent linkage of ubiquitin molecules to one or more lysine residues of a protein. The process of ubiquitination involves the concerted action of the ubiquitination enzymes: E1 (a ubiquitinactivating enzyme), E2 (a ubiquitin-conjugating enzymes), and E3 (a ubiquitin ligases). Atrogin-1 was identified as a muscle-specific E3 that was highly expressed in muscle atrophy (Gomes et al., 2001; Bodine 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 barely expressed in the muscle and liver of chickens. In conclusion, this study shows that feeding *A .Niger* improves growth performance and decreases protein breakdown and could be used as an effective probiotic in broiler chickens.

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