Original Articles

Effect of green tea catechin supplementation in diets for pigs on ammonia emissions from urine and feces mixture

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Abstract The present study aimed to investigate the effects of green tea catechin supplementation to the feed of fattening pigs on the nitrogen level of urine, fecal ammonia emission, and microbial flora. Two diets were used: a control (no green tea catechin added) and a 0.4% green tea catechin-added feed. Eight crossbreed (Meishan×Duloc×Berksher) growing pigs were used in this study. During the growing and fattening periods, feces and urine were collected from each pig to prepare the mixture at a ratio of 1:1 in weight of feces and urine, was placed in a polyethylene sample bag modified with a rubber tube. Each bag was sealed after vacuum suction and inflated with 6 liters of air. The bag was kept at 30±2℃ for 0,6,12,18, and 24 hrs and triplications were prepared for each time. Ammonia gas concentration was determined with Kitagawa precision gas detector tubes. The pH, total nitrogen, and urea concentration in urine and microbial flora in feces were also determined. There was no significant difference in the chemical analysis and microbial flora; however, total nitrogen and urea concentration in urine decreased in the experimental group. Ammonia gas emission in the experimental group was significantly ($P<0.01$ and/or 0.05) reduced in comparison with that in the control group, in regardless of the growth stage. Catechin may have accelerated the utilization of endogenous nitrogen, and reduced the concentration of urea in the urine resulting reduction of ammonia emission from the mixture of feces and urine.

Key words: green tea catechin, ammonia emission, pig

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Introduction

Green tea components have been considered to have pharmacological effects on anti-carcinogenesis [1], anti-mutation [2] and anti-oxidation [3], and various experiments have been conducted to evaluate its role on the growth performance and meat quality in finishing pigs [4-6]. Green tea wastes and green tea by-products have been used in actual experiments. Green tea includes various polyphenol: tannin is one polyphenol extracted from tea leaves and it reduces the palatability of feed. Supplementation of green tea leaves to feed is limited. Besides

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being a major polyphenol, tea catechin has physiologic modulative activities. In experiments to evaluate green tea on the growth performance of pigs, catechin's role as an antibiotic for intestinal microbial flora was expected. The composition of microbial flora is influenced by factors such as diet and feeding conditions, therefore it can easily be estimated that catechin has a supporting and/or reduction effect on microbial proliferation in the digestive tracts of animals. Ammonia is a source of major fecal odor and is a main component of unfavorable odors. Ammonia is synthesized through the reaction of urea in urine and urease in microbial feces, therefore the ammonia emission level is due to the amount of urea and activity of urease. Although the effect of several feed ingredients on ammonia emission has been observed in earlier studies, there is limited information available about the experimental use of catechin as a feed supplement [7,8].

In this study, we investigated the effects of green tea catechin supplementation to the feed of fattening pigs on the nitrogen level of urine, fecal ammonia emission, and microbial flora.

Materials and methods

Animal and experimental plan

A total of 8 crossbreed (Meishan×Duloc×Berksher) growing pigs with an average initial body weight of 30.6±0.8 kg were housed in concrete floor pens (5 ㎡/head). The pigs were assigned to two dietary treatments. Each treatment had 4 replications with 4 pigs per replication. The two dietary treatments were the control (no green tea catechin added) and feed containing 0.4% green tea catechin. The basal feed are two types of commercial one (Best Grower and Best Pork; produced by Toyohashi Feed Co.,Ltd., Aichi) and the nutrient composition was adapted to requirements for growth stage. Green tea catechin was purchased from Mitsui Nohrin Co.Ltd. (Tokyo), with a guaranteed purity of over 80 % (detail was shown in Table 1).

Table 1. Details of used green tea catechin

guaranteed level %(w/w)	
epigallocatechin (EGC)	0.6
epiccatechin (EC)	0.2
gallocatechin (GC)	0.0
catechin （C）	0.2
epigallocatechin gallate (EGCg)	58.4
epicatechin gallate (Ecg)	13.8
gallocatechin gallate (GCg)	3.0
catechin gallate (Cg)	0.5
Total	76.7

The appropriate volume of feed was supplied twice a day and drinking water was supplied *ad libitum*. After 6 weeks from the start of experimental feeding in both growing and fattening periods, feces and urine were collected from each pig and used for the following analysis and experiment (Fig.1).

Chemical analysis and measurements of ammonia gas

Total nitrogen in urine used in this study was analyzed by the Kjeldahl method and urea was determined with an analysis kit (Funakoshi Chemical, Tokyo) and spectrophotometer UV-1800 (Shimadzu, Kyoto). The pH of each sample centrifuged at 3000 rpm was determined with a pH

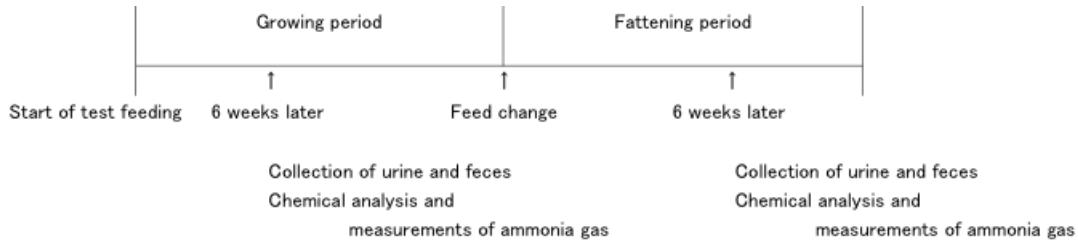

Fig.1 Experimental schedule

meter (DKK Toa Co., Tokyo), the determination of urine pH was directly conducted, but for the feces sample it was conducted after the dilution of feces with distilled water in a ratio of 1:4.

Twenty grams of urine and feces mixture, with a mixing ratio of 1:1 from each pig was placed in a polyethylene sample bag modified with a rubber tube. Three bags were prepared for each determination time, respectively. Each bag was sealed by vacuum suction and inflated with 6 liters of air. The bag was kept at 30±2℃ for 0,6,12,18, and 24 hrs. After treatment, ammonia gas concentration was determined with Kitagawa precision gas detector tubes for ammonia (SD type).

Fecal microbial analysis

Fecal microflora were analyzed by PCR-denaturing gradient gel electrophoresis (DGGE) using DCode system (BioRad, Tokyo) with a partial 16S rRNA gene. Genomic DNA from feces was prepared by the methods described by Morita et al. [9]. The DNA was used as template for PCR amplification of a partial 16S rRNA gene with GC-357F and 518R primer pair; PCR mixture was composed 20ml of 2xEmerald Amp PCR master mix (Takara-Bio, Shiga), 2 ml of 10 mM each primer, 50 ng of purified DNA, and up to 40 ml of sterilized water, and thermal condition was according to the method described by Muyzer et al. [10] using touch down methods for annealing step. After PCR 5 ml of the reaction mixture were added with 2 ml of 70% glycerol solution, and then all of the mixture was applied to DGGE gel. DGGE was conducted under 130V for 5 hours at 60ºC with from 30 to 60 % of denaturant and 8% acrylamide gel. 100% denaturant was including 7 M urea and 40% formamide. After the electrophoresis, the gel was stained with SYBR Gold (Invitrogen, Tokyo), and then band pattern was visualized LAS-4000 image analyzer (Fuji Firm, Tokyo). Band pattern was compared to previous results, which were sequenced and identified by Genus level [11]. Band concentrations were measured by Image J software (http://rsbweb.nih.gov/ij/) and compared by relative amount at same position in each lane.

Statistical treatment

Ammonia gas emission from each treated group after the determined period was compared by one-way ANOVA using Excel statistical software at significant level of $P<0.05$ and 0.01. Mean separation

Table 2.Chemical characteristics of urine and feces

		Growing		Fattening	
		Control	Experiment	Control	Experiment
Total nitrogenin urine	(%)	0.83±0.15	0.70±0.05	1.06±0.14	0.99±0.05
Urea in urine	(mg/mL)	0.39±0.10	0.31±0.01	0.44±0.02	0.39±0.02
pH urine		7.85±0.3	8.07±0.3	7.75±0.3	7.40±0.4
feces		6.21±0.1	5.92±0.1	6.22±0.2	6.16±0.1

Mean±S.E., control group n=3, Experiment group n=4.

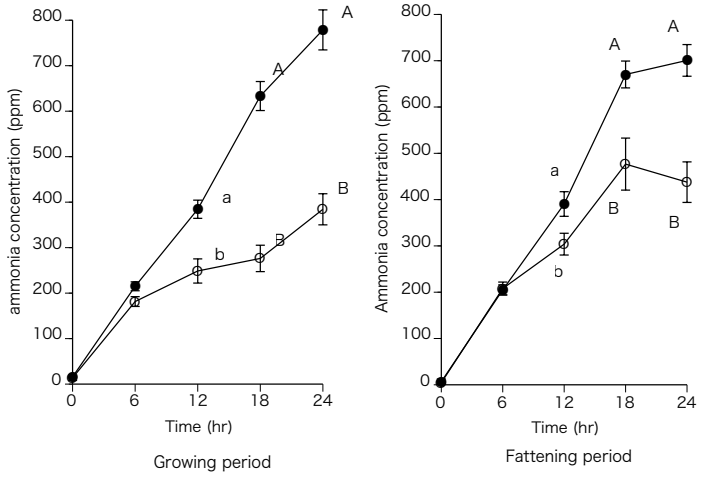

Fig.2 Ammonia emission from the mixture of feces and urine collected from pigs fed with (○) or without (●) catechin

Significant difference was recognized between the different superscript letters; A,B: P<0.01, a,b: P<0.05.

among the test groups was done using the Least Significant Difference method.

Results

Chemical analysis and measurements of ammonia gas

The results of chemical analysis are shown in Table 2. There is no significant difference; however, total nitrogen concentrations in the control group were larger than those in the experimental group in both collection periods, growing and fattening. Concentrations of urea in urine from the experimental group pigs were smaller at 88-80% than those in the control group, though there was no significant difference (P<0.19). Moreover, there was no definite tendency in the pH values of urine and feces.

Ammonia gas emission from the mixture of urine and feces that were collected from the growing period and the fattening period were observed for 24 hrs (Fig.2). Ammonia concentrations emitted from the mixture of the experimental group of pigs fed green tea catechin was significantly (P<0.05 or 0.01) lower than those of the control group at 12, 18 and 24 hr of incubation.

Fecal microbial analysis

The number of fecal microorganisms was never determined in this study. The only classification of microorganism strain was conducted by the DNA isolation method, and the result is shown in Table 3. Recognized microorganisms included the following 6 strains: *Lactobacillus, Clostridium, Streptococcus, Psychrobacter, Escherichia,* and *Megasohaera*. Slight differences were recognized between the classification rate of each microorganism in the control and experiment groups, but there was no significant difference.

Table 3 Results of fecal microorganism classification rate(%) by DNA isolation method from pig feces

Strain	Growing period		Fattening period	
	Control n=3	Experiment n=4	Control n=3	Experiment n=2
Lactobacillus	6.8±1.0	8.0±0.9	8.1±0.6	6.6±0.2
Clostridium	38.3±3.3	36.6±2.1	38.3±2.9	42.0±0.1
Streptococcus	21.7±1.6	25.5±1.6	21.6±1.9	21.6±5.0
Psychrobacter	7.6±1.7	4.7±1.6	3.8±0.6	1.8±0.2
Escherichia	4.1±0.8	3.0±0.7	1.4±0.0	trace
Megasohaera	0.4±0.0	2.1±1.2	5.0±1.0	4.1±0.0
Other	21.1±1.9	20.2±2.0	22.7±0.8	23.9±5.0

Mean±SE

Classification rate was calculated for total strain recognized in the present isolation sample.

Discussion

Bacteria in the digestive tracts is easily affected by feed composition, such as dietary fiber [12,13] or physiologic functional substances like a polyphenol and it is easily deduced that the change of microbial flora influences the odor and ammonia emissions [14,15]. Furthermore the microbial flora alters its population in accordance with the passage of exposed time [16]. In an earlier study [17], the effect of tea polyphenols on the fecal ammonia emission and microbial flora was evaluated with 30-day-old piglets: increases in the *Lactobacilli* level and decreases in the *Bacteroidaceae* level and ammonia emission were reported. The present results which used growing-finishing pigs showed the same effect on the reduction of ammonia emissions, but there was no remarkable difference in the species of microbial flora. Lower ammonia emission in green tea catechin feed was observed in this study, which is not consistent with the results of microbial flora determination: it in somewhat consistent with the slight difference in the nitrogen level in urine. The relation between ammonia emission and total nitrogen level in urine were coefficients in both growth stage. The same relation between ammonia gas emission and total nitrogen in urine and/or urine level was reported in a previous experiment [18,19]. Furthermore in this study we determined the urea amounts in urine decreased in the experimental group. There was no significant difference in the total nitrogen and urea in urine as shown in Table 2, but those values in the experimental group showed decrease tendency. The ammonia emission were decreased significantly by the using of experimental group urine as shown in Fig.2. The present tendency was sustained by the knowledge in the earlier report, in which recognized the coefficient relation between the ammonia emission and the nitrogen level in the feces-urine mixture [19]. Green tea catechin intake may influence microbial flora, but it was not so

remarkable during the growing-finishing stage of pigs, and to it a degree, may influence nitrogen metabolism in increasing the efficient utilization due to the decrease of urea in urine. Although the results of growth performance for the tested pigs were not described in the present report, the feed efficiency in the experiment group fed green tea catechin tended to be higher than that of the control group. This tendency suggests that high utilization of nitrogen by catechin in the experimental group. The physiological mechanism of this remains to be elucidated in a future work.

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原　著

豚用飼料への茶カテキン添加がふん尿混合物からの アンモニア揮散に及ぼす影響

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本試験は肥育豚飼料への緑茶カテキン（以降カテキン）添加が、ふん尿から揮散するアンモニアの濃度に及ぼす影響について検討した。対照区はカテキン無添加の市販飼料、試験区は対照区飼料にカテキン 0.4%を外付け添加した飼料を準備した。8 頭の三元交雑種（MDB：梅山豚×デュロック×バークシャー）を平均体重が等しくなるように 4 頭ずつ配分し、試験飼料を給与した。給与開始から 6 週間後に糞と尿を個別に採取し、糞尿を 1:1 の割合で混合してゴム管を付けたポリエチレン製のサンプル袋に 20g ずつ入れ、中の空気を排出した。6L の空気を注入後、30±2℃の空調室で、試験開始から 24 時間後まで 6 時間ごとにアンモニア揮散量を北川式ガス検知管で測定した。糞尿の pH、尿中の全窒素量、尿素量、糞中微生物叢の検索も行った。アンモニア揮散量は 12 時間後に対照区に比べて試験区が有意($P<0.05$)に低くなり、その差は 18 時間以降に顕著になった($P<0.01$)。糞尿の pH に一定の傾向は見られず、尿中の全窒素量に有意な差は無かったが、試験区が対照区より少なかった。また、尿素量は試験区が対照区の 88-80%程度に低かった。糞便から抽出・精製した微生物の 16S リボソーム RNA 遺伝子を PCR 法にて増幅後、変性剤濃度勾配ゲル電気泳動（DGGE）法に供し、得られた 6 つのバンドについて、DNA を抽出・精製し 357F−518R プライマーペアで PCR 増幅後、両方向配列解析し、BLAST 検索して属名を決定した。またバンドの濃淡で相対的な存在比を比較したが、6 系統の細菌割合に区間の差は認められなかった。よって、カテキン摂取によるアンモニア揮散量の差は豚の体内における窒素利用が高まり、尿中へ尿素として排泄される量が低減した事に起因すると考えられた。

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