Inhibitory Effect and Mechanism of Whole Grain Paddy Rice Feeding on *Campylobacter* Colonization in the Cecum of Broiler Chicks

（籾米給与によるブロイラー盲腸でのカンピロバクター定着抑制とそのメカニズムの解明）

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Chapter 1 General introduction

Campylobacter: characteristics and pathogenesis

Campylobacter spp. bacteria are curved, spiral- or S-shaped Gram-negative rods (0.2–0.8 μm wide and 0.5–5.0 μm long) that do not form spores (Griffiths and Park, 1990; Ottosson and Stenström, 2000). They achieve optimal growth at pH 6.5–7.5 but cannot survive below pH 4.9 or above pH 9.0. As they are generally microaerophilic, they cannot survive under normal atmospheric conditions. However, *Campylobacter* spp. can be cultured in atmospheres with 3%–15% oxygen, supplemented with 2%–10% CO_2 (Forsythe, 2000). Thermophilic *Campylobacter* spp. can grow between 37°C and 42°C but not below 30°C (Van de Giessen *et al*., 1996).

Campylobacter spp. are major pathogens of bacterial enteritis in humans and are responsible for 400–500 million cases of human infection around the world each year (Ruiz-Palacios, 2007). An infective dose of *Campylobacter* spp. is as low as 500 colony forming units (cfu) **(**Medema *et al*., 1996**;** Robinson, 1981). On infection in the host, the major prominent clinical manifestation in humans usually involves a self-limited gastrointestinal illness lasting up to 7 days followed by an incubation period of 1–7 days (Altekruse *et al*., 1998; Pebody *et al*., 1997). Campylobacteriosis is characterized by a longer incubation period than that of the other bacterial enteritides and requires fewer cfu to cause infection. Clinical manifestations of campylobacteriosis are extremely diverse, ranging from a complete absence of symptoms to fulminating sepsis and rarely death, mainly in immunosusceptible hosts (Hong *et al*., 2004). *Campylobacter* infections are also associated with postinfectious complications including arthritis, Reiter syndrome, and Guillain–Barré syndrome (Hong *et al*., 2004). Guillain–Barré

syndrome, the prototype of postinfectious autoimmune diseases, is characterized by acute onset of limb weakness and loss of tendon reflex (Takahashi *et al*., 2005).

Campylobacter was identified for the first time in 1906 when two British veterinarians reported the presence of "large numbers of a peculiar organism" in the uterine mucus of a pregnant sheep (Skirrow *et al*., 2006). Since then, *Campylobacter* spp. are known to cause spontaneous abortion in livestock. In 1973, they were recognized as human pathogens (Butzler, 2004). In Japan, they were isolated and reported as pathogens for the first time in 1979 (Itoh *et al*., 1980) and were registered as food-poisoning bacteria in 1982. *Campylobacter* spp. were classified into 23 species in 2011; the most frequently detected species *C. jejuni* is responsible for more than 95% of cases of *Campylobacter* infection.

Campylobacter spp. are a part of normal bacterial flora in many types of animals (livestock such as cattle, pigs, and chickens; companion animals such as dogs and cats; and wild animals such as birds). Among *Campylobacter* spp., *C. jejuni* and *C. coli*, which cause food poisoning, are carried by some types of domesticated animals; chickens, cattle, and sheep carry *C. jejuni*, and pigs carry *C. coli* in their intestinal tracts. These animals are recognized as the main hosts and the source of human infection by the bacteria. Ingestion of foods that are contaminated by the bacteria or through contact with carrier animals causes the infections in humans. Many cases of campylobacteriosis occur in developing countries because of the consumption of *Campylobacter*-contaminated river water as drinking water.

Campylobacter **food poisoning**

In recent years, although the overall trend in Japan is toward a decrease in the

number of cases of bacterial food poisoning, the number of cases and patients with *Campylobacter* food poisoning are on the increase (MHLW, 2009). However, in most cases of *Campylobacter* food poisoning, the implicated food is not identified. Among the reasons for the difficulty in identifying the suspicious food are two in particular. First, campylobacteriosis has a relatively longer latent period (2–7 days) than other bacterial infections; thus, foods under suspicion as cause of infections will have already been consumed or disposed of. Second, because *Campylobacter* organisms are microaerophilic, the bacteria quickly die under atmospheric oxygen content of over 15%, making it difficult to detect the bacteria in the suspicious foods. According to the risk assessment report of the Food Safety Commission of Japan, chicken meat and chicken meat dishes account for 40% of causative foods identified in *Campylobacter* food poisoning; in particular, chicken meat that is identified as the causative food were highly contaminated with *C. jejuni*, and the contamination rate of Japanese-reared chicken selling at a retail store is 65.8% (FSCJ, 2009).

It is generally believed that chicken meat is contaminated with *C. jejuni* during chicken meat processing. The average rate of contamination of poultry farms with *Campylobacter* is reported to be 11%–78% (median of 57.9%, according to the Food Safety Commission of Japan), an indication that all chickens bred for the market are not infected with *Campylobacter*. However, live chickens infected with *Campylobacter* can cross-contaminate other chickens in meat-processing plants, consequently raising the rate of *Campylobacter*-contaminated chicken meat reaching the market (Misawa *et al*., 2012), so that the average rate of contamination of domestic chicken meat increases to 32%–96% (median of 75%) (Food Safety Commission of Japan).

The main causes of the spread of *Campylobacter* contamination in chicken

meat-processing plants are discussed next. First, meat is processed in the plant without separating each carcass from the others. When, as often happens during processing, the intestines tear, the bacteria enter the water used in the processing. Second, sodium hypochlorite, which is officially permitted for use in chicken meat processing as a sanitizer, is not necessarily effective against *Campylobacter* adhering to carcasses (Misawa *et al*., 2012). Considering the present conditions of production, transport, and processing of chicken meat described above, it is very important to establish an effective strategy that can decrease the number of *Campylobacter* carrier birds at the farming stage so as to prevent *Campylobacter* food poisoning. It has been reported that decrease in *Campylobacter* spp. colonization in broilers on the farm may decrease the infectious loads entering abattoirs and; thus, decrease the amount of *Campylobacter*-contaminated chicken meat (Ellis-Iversen *et al*., 2009). Rosenquist *et al.* (2003) also reported that a 2-log reduction in *C. jejuni* numbers on chicken carcasses can lead to a 30 times lower risk of human campylobacteriosis.

Routes of *Campylobacter* infection of farm-reared chickens

In general'flocks remain negative for *Campylobacter* for up to 2-3 weeks age (Bull *et al*., 2006; Jacobs-reitsma *et al*., 1995). As described above, the average rate of contamination of poultry farms with *Campylobacter* is reported to be 11%–78%, and the rate of contamination of chickens is high (median 84.5%) in the contaminated poultry farms (Food Safety Commission of Japan). *Campylobacter* organisms infect via oral ingestion and predominantly colonize the mucus layer of cecal crypts (Beery *et al*., 1988; Meade *et al*., 2009; Newell *et al*., 2003). Chickens are so susceptible to

colonization by *Campylobacter* that inoculation doses as low as 40 cfu result in cecal colonization (Cawthraw *et al*., 1996). Once a chicken's digestive tract has been colonized with *Campylobacter*, the chickens carry large numbers of the bacteria in their cecum (generally approximately 10^5–10^9 cfu/g) and remain colonized until slaughtered (Berndtson *et al*., 1992; Berrang *et al*., 2000). As broilers are usually reared in large flocks, once *Campylobacter* organisms have been introduced, the infection spreads within a few days throughout the broiler house. Drinking water and feed are believed to play an important role in the fecal-to-oral transmission within the flock (Berndtson *et al*., 1996; Newell and Fearnley, 2003; Sparks, 2009). The route of *Campylobacter* invasion from outside of poultry houses has not been epidemiologically confirmed. However, as *Campylobacter* organisms have been isolated from water supplies, rat feces, and soil around poultry houses, it has been presumed that rodents, insects, dust, and environmental water carry the bacteria into the poultry house (Hald *et al*., 2004, 2008; Hazeleger *et al*., 2008). In addition, the ventilation provided to condition the temperature and humidity in a poultry house facilitates the entry dust and insects into the house through the wall openings.

Measures against *Campylobacter* food poisoning

Current broiler breeding systems, in which chickens are raised in large flocks with high density and a ventilation system lacking bacterial filtration, face extreme difficulties breeding broiler chickens without being invaded by *Campylobacter* organisms from outside the farm. As a practical preventive measure, *Campylobacter* colonization in chicken digestive tracts might be suppressed by feed additives such as antibiotics. However, according to the Law Concerning Safety Assurance and Quality

Improvement of Feeds, feeding of antibiotics is only permitted for 7 days before shipment for slaughter. In addition, feeding antibiotics to chickens has become hazardous because of the global emergency of antibiotic resistance in *Campylobacter*; the World Health Organization has recognized it as a public health problem (Greig, 2003; McDermott *et al*., 2005; Moore *et al*., 2006). This untenable situation leads us to propose a strategy to prevent *Campylobacter* colonization via oral invasion through introduction of natural feed additives that enhance the digestive function of individual chickens to protect against pathogenesis. Our strategy is described next.

A proposed strategy to protect farm-raised poultry from *Campylobacter* infection

Chicken digestive tracts feature a diverticulum, called "the crop," in part of the esophagus, wherein ingested foods are stored temporarily. *Lactobacillus* spp., the predominant flora in the crop (Rehman *et al*., 2007), produce organic acids and bacteriocins that display bactericidal or bacteriostatic activity against pathogenic bacteria entering via the oral route (Fuller, 1973; Jin *et al*., 1996). The proventriculus and the gizzard have functions equivalent to that of the human stomach, wherein hydrochloric acid and pepsinogen are secreted by the proventriculus and are mixed with the contents from the crop by the gizzard's grinding activity. As the inside of the gizzard is maintained at a low pH because of hydrochloric acid secreted from the proventriculus, the gizzard could function as a bacteriostatic organ that protects against bacteria invading via the oral route. Therefore, an enhancement of these protective functions against pathogens of the crop and the gizzard in individual chickens could be a promising strategy to prevent the pathogenic bacteria from infecting the upper gastrointestinal tract via oral ingestion.

Measures to enhance the natural defense function in chickens

One of the materials that show promise for enhancing the natural defense function of the upper gastrointestinal tract of chickens is insoluble fibers. These fibers are plant cell wall polysaccharides, such as cellulose and lignin, which have effectively promoted peristalsis in the human intestinal tract and are recommended as health-promoting materials to be introduced in human nutrition. In contrast, insoluble fiber has not been recognized as an important feedstuff for chicken breeding. These plant cell wall components can be expected to have a similar effect in chicken digestive tracts to that for humans, because insoluble fiber components resist breakdown by 1) promoting longer retention time in the crop and 2) enhancing gizzard activity. These effects on the upper intestinal tract could result in an extension of the food contact time with the acidic gastric juice that suppresses bacterial growth in these organs. Santos *et al.* (2008) suggested the possibility that gizzard activity promoted by stiff food components could suppress the growth of pathogenic bacteria entering via the oral route.

Several studies have reported on the effectiveness of the stiff plant cell wall components described above for the prevention of *Campylobacter* infection in broiler chickens. Moen *et al*. (2012) reported that the spread of *Campylobacter* infection in flocks was delayed by feeding broilers with a diet composed of 15% dietary fiber (7.5% oat hull, 7.5% barley hull) beginning at 10 days of age. Skånseng *et al*. (2013) reported that the spread of *Campylobacter* in a breeding group was delayed by a few days (as compared to control chickens) in chickens whose feed contained 12% oat hulls.

For this study the author selected paddy rice as the feed ingredient including dietary fiber to suppress *Campylobacter* infection. Rice has been widely cultivated in the regions including Southeast Asia and East Asia, and it has nourished much of the

population of this region for more than 2000 years. Japan has 2.46 million ha of rice fields that have the potential to produce several million tons of rice annually (Ministry of Agriculture Forestry and Fisheries statistics).

Paddy rice is spindle-shaped with a length of approximately 10 mm and an average diameter of 3 mm. Because paddy rice is smaller than either barley or oats, broiler chickens of more than 14 days of age could easily consume it. Addition of paddy rice (i.e., unprocessed rice, unhulled) to the poultry diet has the advantage over other natural foodstuffs of easily supplying stiff dietary fiber to chickens while acting as an energy source as well. Because some types of dietary fiber are unpalatable to chickens, the birds tend to selectively avoid eating them when mixed into the feed by itself. Chickens could unintentionally eat the dietary fiber that corresponds to approximately 20% of rice through paddy rice feeding, ingesting 12 g dietary fiber per 60 g of paddy rice in the feed.

Scope and content of this doctoral thesis

This thesis describes a study conducted by the author to test the feasibility of feeding whole-grain paddy rice to farm-raised broiler chickens to prevent the infiltration of *Campylobacter* spp. into the upper intestinal tract with the goal of enhancing the natural bactericidal barriers inherent in the birds' anatomy.

Chapter 1 provides a general introduction to this thesis.

Chapter 2 presents the author's finding that feeding of whole-grain paddy rice inhibited the dosed *C. jejuni* from colonizing the cecum of broiler chicks.

Chapter 3 describes the proposed mechanism involved in the inhibitory effect of paddy rice feeding on *Campylobacter* colonization in broiler chickens. In short, paddy

rice feeding affords both a longer retention time in the crop and a uniform internal pH if the gizzard, an effect that might suppress *Campylobacter* growth in the gastrointestinal tract of broiler chicks.

Chapter 4 summarizes the content of the thesis.

Chapter 5 presents some concluding remarks.

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Chapter 2 Inhibitory Effect of Whole-grain Paddy Rice Feeding on the Colonization of *Campylobacter jejuni* in the Cecum of Broiler Chicks

Abstract

To examine the inhibitory effect of whole grain paddy rice diet (WPR) feeding on the colonization of *Campylobacter jejuni* in the cecum of broiler chicks, we performed the following examination. Sixty female broiler chicks (14 days of age) were allocated into two groups, thirty birds were fed a ground corn diet (GC) as a control group, and 30 chicks were fed WPR as an examination group. After feeding with the different diets for 16 days, six chicks were selected from each group (12 chicks in total), and each chick was inoculated with 2×10^3 colony-forming units of *C. jejuni* GTC03263. Forty-eight hours after the bacterial inoculation (the birds continued to be fed with the corresponding diets after the bacterial inoculation), the chicks (six from each group) were killed and dissected to detect bacterial colonization in the cecum. The other six chicks were dissected to weigh the chick gizzard and to measure the pH of its contents. No bacterial colonization was observed in the cecum of chicks in the WPR group, whereas bacteria were found in the cecum of five of six chicks in the GC group. The average ratios of gizzard weight to body weight of the WPR-fed chicks was significantly higher than that of the GC-fed chicks, whereas the average pH value of the gizzard contents was not different between the two groups. These results suggest that WPR feeding in broiler chicks has a positive effect on development of the gizzard muscle and grinding activity of the gizzard. Increased grinding activity may eliminate the regional differences in pH within the gizzard, resulting in less bacterial survival in

the gizzard and then less bacterial colonization in the cecum of WPR-fed chicks than in GC-fed chicks.

Introduction

Campylobacter jejuni is one of the most serious pathogenic causes of food-borne gastroenteritis in humans, and the infection is a public health problem in many countries (Blaser, 1997; Silva *et al*., 2011). Hermans *et al.* (2012) revealed that poultry is the most suspicious infection source for human food-borne gastroenteritis. Broiler chickens are commonly regarded as natural hosts for this zoonotic pathogen, and they are rarely infected at younger than 1 week old (most flocks become infected when they are 3–4 weeks of age, Evans and Sayers, 2000; Hermans *et al*., 2012). Chickens have several natural barriers to infectious pathogens in the upper gastrointestinal tract. The crop contains lactic acid bacteria (Hilmi *et al*., 2007). Meanwhile, the gizzard contains hydrochloric acid to facilitate digestion of the feed, and it may also have a sterilizing effect by which food pathogens are potentially killed in a time-dependent manner, in the acidic environment (Engberg *et al.*, 2002). In addition an ingredient of coarse particles such as whole wheat (Bjerrum *et al*., 2005), coarsely ground corn, and whole triticale (Santos *et al*., 2008) have been reported to reduce the frequency of *Salmonella* infection in young broilers. The increase in the content of oat and barley hulls (Moen *et al.*, 2012), as well as whole wheat and oat hulls (Skånseng *et al*., 2013), in the feed delays the horizontal spread of *C. jejuni* in broiler flock. Bjerrum *et al*. (2005) suggested that the reduction in the frequency of *Salmonella* infection in birds fed a diet containing whole wheat was caused by the combinational effect of a lower pH and longer retention time of the pathogens in the gizzard. These aforementioned reports indicated that the whole

grain or coarse particles in chicken feed play a role in preventing pathogenic bacteria from invading the chicken gastrointestinal tract.

Rice is the most staple grain in East and Southeast Asia, and dehulled rice and whole grain paddy rice are widely served to broiler chickens as an energy source. Whole grain paddy rice has particle size similar to that of whole wheat (Solà-Oriol *et al*., 2009), and it is covered with rice hull similarly as oat hull. The morphological similarities among whole grain paddy rice, whole wheat, and whole oat prompted us to investigate the inhibitory effect of feeding with a whole grain paddy rice diet (WPR) on pathogenic bacterial infection in broiler chicks. The report of feeding with dehulled rice and WPR has not been published from the viewpoint of their inhibitory effects on bacterial infection in broiler chicks. The objective of this study was to examine whether WPR feeding prevents the colonization of C*ampylobacter* in the cecum, which is the principal site of *Campylobacter* colonization in the lower gastrointestinal tract of chickens (Beery *et al*., 1988; Stern *et al*., 1988; Meinersmann *et al*., 1991; Achen *et al*., 1998; Hermans *et al*., 2012).

Materials and Methods

Animal care

The procedures involving animals and their care conformed to the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006).

Chick rearing and feeding

Sixty 1-day-old female broiler chicks (chunky) had been raised in a flock rearing area, which was approximately 2 m^2 and spread out with sawdust, in a windowless broiler

house. The rearing area temperature was controlled at 34°C on the first day of rearing and gradually lowered to 25°C until the chicks reached 14 days of age. After 15 days of age, the room temperature was kept between 20 and 25°C. The chicks were given a starter feed until 14 days of age (Table 1). This feed contained ground corn that had been crushed into particles of less than 1.4 mm in size (68.5% as a weight ratio). On day 14, the chicks were allocated into two groups, ground corn diet (GC) fed group or WPR fed group, of which the main ingredients were 75% ground corn and 65% whole grain paddy rice, respectively (Table 1). Ground corn was supplied by Yao Feed Co., Ltd, Osaka, and whole grain paddy rice (Momiroman) was obtained from Kyoto Prefectural Agriculture Experiment Station. The particle size distribution of ground corn, GC, whole grain paddy rice, and WPR was determined by the dry sieving method as summarized in Table 2. The feedstuffs of the feed did not contain any antimicrobials or coccidiostats. All chicks had free access to feed and water throughout the trial. The duration of light exposure was controlled continuously until 7 days of age, and the duration of light exposure was 20 h per day after the birds reached 8 days of age. During the experiment birds' feed intake and body weight were measured weekly.

Measurement of weight of the gizzard and pH of its contents

At 28 days of age, 12 chicks from each flock were randomly selected and weighed, and then they were killed by suffocation with CO_2 gas. The gizzard of each bird was removed and incised with surgical scissors. Then, the contents of the gizzard were removed and added to two volumes of deionized water, and the mixture was mixed using a vortex mixer for 1 min in a 50-ml centrifuge tube. The pH of the diluted gizzard contents was measured using a glass pH meter (9615-10D, HORIBA) twice for each

sample. The remained gizzard was washed with water, and empty gizzards were weighed.

Inoculation and bacterial count in the cecum

At 25 days of age, six chicks were selected from each flock and caged in three experimental cages (two chicks per cage). The birds were fed with their corresponding feed during this time. Before the bacterial inoculation, the cloaca of each chick (30 days of age) was determined to be free of *Campylobacter* spp. using the cloacal swab method. *C. jejuni* GTC 03263 was pre-cultured on brain heart infusion agar (Nissui Pharmaceutical Co., Ltd) at 42°C for 48 h under microaerophilic conditions (85% N_2, 10% CO_2, and 5% O_2). One platinum loop of the pre-cultured bacteria was suspended in 5 ml of sterilized saline (suspension contained approximately 1×10^8 colony-forming units [cfu]/ml) and serially diluted in sterilized saline to a final concentration of 2×10^3 cfu/ml. Twelve chicks (30 days of age) in the experiment cages were inoculated with 2×10^3 cfu of *C. jejuni.* The inoculation was performed individually for each bird via the crop instillation of 1 ml of the bacterial suspension using a 1-ml syringe with an attached flexible tube.

Forty-eight hours after the inoculation (chicks were 32 days of age), all chicks were killed by suffocation with CO_2 gas. The cecum was removed, and the contents were transported into 15-ml tubes individually and kept in an icebox for 2 h before bacterial detection.

One gram of the cecum contents removed from the chicks was suspended and mixed with a dilution solution for anaerobes (composition in 1000 ml of purified water: KH_2PO_4, 4.5 g; Na_2HPO_4, 6.0 g; L-cysteine hydrochloride, 0.5 g; Tween 80, 0.5 g; agar, 1 g; Mituoka, 1994), and then the suspension was plated onto modified charcoal

cefoperazone desoxycholate agar (CCDA) plates (Oxoid, Basingstoke, United Kingdom). CCDA consisted of a campylobacter blood-free selective agar base (Oxoid CM739) with *Campylobacter* selective supplement (Oxoid SR155) and *Campylobacter* growth supplement (Oxoid SR084). The plates were incubated at 42°C under microaerophilic conditions (85% N_2, 10% CO_2, and 5% O_2) for 48 h. One gram of the remaining cecum contents was added to 10 ml of Preston broth (Oxoid) and cultured at 42°C for 24 h. One platinum loop of the Preston broth was then plated onto a CCDA plate. The plates were incubated at 42°C under microaerophilic conditions (85% N_2, 10% CO_2, and 5% O_2) for 48 h. Some colonies suspected to be *C. jejuni* on the plate were isolated and incubated on blood agar base no. 2 (Oxoid) containing 5% defibrinated horse blood. The isolated colonies were confirmed to be *C. jejuni* using standard microbiological methods (International Standards Organization, 2006), including Gram stating, catalase and oxidase tests, specific spiral morphology, and corkscrew motility observed by phase-contrast microscopy.

Statistical analysis

Statistical comparison of the average values of the chicks' body weight, the ratio of the removed gizzard weight to the chick body weight was performed using Student's *t*-test. The comparison of the pH values of the gizzards was also performed using Student's *t*-test (two-sided). The incidence of *Campylobacter* infection in the chicks was analyzed by Fisher's exact test. *P*-values less than 0.01 were considered statistically significant. Both tests were conducted using Microsoft Excel 2010 add-in software.

Results

Table 3 shows chicks' feed intake and body weight during the experiment. Statistical analysis showed that there was no significant difference between the two groups in initial and final body weight.

Table 4 presents the average gizzard weight/100g body weight ratios of the WPR- and GC-fed chicks (mean ± SD) at 28 days of age. Statistical analysis (*t*-test) revealed that the gizzard weight/body weight ratio of the chicks fed WPR was significantly higher than that of the chicks fed GC ($p = 0.00031$). Table 4 reveals that average pH values of the contents of the gizzard were not different significantly between the two groups ($p = 0.21458$). Table 5 shows the results of the detection of *Campylobacter* in the cecum of chicks inoculated with bacteria followed by feeding with WPR or GC. *Campylobacter* was not detected in the cecum of WPR-fed chicks, whereas *Campylobacter* was detected and identified in the cecum removed from five of six chicks in the GC group. Statistical analysis revealed a significant difference in the incidence of *Campylobacter* infection between the different feeds ($p = 0.00758$).

Discussion

The gizzard is a muscular organ that reduces the particle size of ingested foods and mixes them with digestive enzymes (Duke, 1986). The inclusion of whole wheat, coarse ground maize, oat hulls, barley hulls, and wood shavings in poultry diets have been demonstrated to modify the upper gastrointestinal tract, resulting in increased gizzard weights (Svihus, 2011), as coarse feed particles need to be ground to a certain critical size before they can exit the gizzard (Svihus, 2011). Concerning the size of particles that can exit the gizzard, Hetland *et al*. (2002) reported that the majority of the particles entering the duodenum are smaller than 100 μm, even when considerable amounts of

whole wheat or coarse cereal particles are added to the diet. Regarding the application of the whole grain paddy rice as a coarse particle ingredient to modify the weight of the gizzard, Sittiya and Yamauchi (2014) and Kita and Okuten (2013) reported whole grain paddy rice feeding in Sanuki Cochin and young chickens, respectively, and observed that the gizzard of chicks fed whole grain paddy rice developed better than that of chicks that were not fed whole grain paddy rice. In this study, we found that the average gizzard weight/body weight ratio of the chicks fed WPR was significantly higher than that of the chicks fed GC, which indicates that WPR feeding in broiler chicks has a positive effect on the development of gizzard of chicks.

As the gizzard is a muscular organ, it is reasonable to suppose that the more gizzard developed, the more gizzard has grinding activity. It is, however, unclear whether enhancement of the gizzard grinding activity promoted by the feeding of coarse feeds affects the pH of its contents. Gabriel *et al.* (2003), and Engberg *et al.* (2004) reported lower pH values in the gizzard contents of birds fed whole wheat, oat hulls and soy hulls (Jiménez-Moreno *et al.*, 2009b), oat and barley hulls (Sacranie *et al.*, 2012), oat hulls and sugar beet pulp (Jiménez-Moreno *et al*., 2009a, 2013), and oat hulls, soybean hulls and sugar beet pulp (Mateos *et al*., 2012), whereas Hetland *et al*. (2002), González *et al*. (2008), Jacobs *et al*. (2010), and Singh *et al*. (2014) reported that no significant effect of the feeding of coarse feeds on the pH of gizzard contents. In this study, we found no significant difference the gizzard content pH between WPR and GC-fed chicks, and this result was similar to Hetland *et al*. (2002), González *et al*. (2008), Jacobs *et al*. (2010), and Singh *et al*. (2014).

Concerning the pH of the gizzard contents of chicks, which influence the possibility of the bacterial survival in the gizzard, the optimum pH for the growth of *C. jejuni* is 6.5–7.5, and the bacterial cell numbers significantly decrease when the pH falls below

4.0 (Jackson *et al*., 2009). In this study, we found that the pH of the gizzard contents were 2.96 and 3.15 in the gizzard fed WPR and fed GC, respectively, which indicates that the dosed *Campylobacter* could be exposed to the bacterial extinction pH level in the both case. However, no bacterial colonization was observed in the cecum of chicks in the WPR group, whereas bacteria were found in the cecum of five of six chicks in the GC group. In this connection, Walk *et al*. (2012) examined the pH in the gizzard as determined via the direct insertion of a pH probe into the proximal and distal gizzard of broiler chickens fed a low-calcium (0.64%) diet and found that the pH values of the proximal and distal gizzard contents were 1.85 and 2.44, respectively. This report indicates that there is regional difference in pH of the gizzard contents. Here, we speculate that the non-uniformity pH in the gizzard could be diminished if the activity of the gizzard is sufficient to mix and homogenize the contents well and ensure pH levels to prevent invasion of the pathogen. We need, however, further experiments to clarify the relationship between the grinding activity of the gizzard and the homogeneity of gizzard pH.

In this study, we found that the gizzard of chicks fed WPR developed more than chicks fed GC and no bacterial colonization was observed in the cecum of chicks in the WPR group, whereas bacteria were found in the cecum of five of six chicks in the GC group. From these results we can propose a hypothesis that may explain our present results; well-functioning gizzard of chicks fed WPR may enhance the probability of "sterilization" of the inoculated *Campylobacter* by the uniform (homogeneous) low pH in the gizzard, in contrast, not-well-functioning gizzard of chicks fed GC could not mix the contents sufficiently well to make uniform low pH in the gizzard.

In conclusion, WPR feeding in broiler chicks has a positive effect on the development of the gizzard muscle and then grinding activity of the gizzard. Increased grinding

activity may eliminate the regional differences in pH within the gizzard, resulting in less bacterial survival in the gizzard and less bacterial colonization in the cecum of WPR-fed chicks than in GC-fed chicks. This result suggests that the gizzard has an important barrier function to prevent pathogenic bacteria from entering the lower intestinal tract via the cooperative action of the activity of the gizzard and the pH of its contents.

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Table 1. **The composition of the experimental diets**

Ingredient(%)	Starter (1 to 14 d)[1]	Grower (14 to 32 d)	
		GC[2]	WPR[3]
Corn	68.50	75.00	9.00
Paddy rice	—	—	65.00
Soybeen meal	17.00	10.50	8.00
Fish meal(CP65%)	7.00	1.50	1.00
Corn gluten meal	3.00	8.00	11.00
Soybean oil	2.00	2.00	3.00
Calcium carbonate	0.65	0.60	0.70
Tricalcium phosphate	0.75	1.40	1.40
Manganese sulfate	0.012	0.013	0.01
Sodium chloride	0.22	0.11	0.17
DL-Methionine	0.10	0.10	0.05
L-Lysine HCl	0.30	0.50	0.60
Riboflavin	0.0005	0.0002	0.0002
Copper sulfate	0.0003	0.0006	0.0005
Zinc sulfate	0	0.001	0.003
Folacin	0.00003	0.00004	0.00002
L-Threonine	0.10	0.15	0.15
Choline chloride	0.03	—	—
Calcium pantothenate	0.0002	—	—
vitamine/mineral premix[4]	0.35	0.25	0.25
Calculated			
Crude protein (%)	20.3	16.2	16.1
Metabolize energy (Kcal/kg)	3031	3087	2883

[1] The starter diet was ground and then passed through a sieve with 1.4-mm mesh.

[2] Ground corn diet.

[3] Whole grain paddy rice diet.

[4] Vitamin and mineral premix including (per kilogram of diet): retinol (retinyl acetate), 3500000 IU; cholecalciferol, 700000 IU; vitamin E (DL-α-tocopheryl acetate), 600 mg; menadione, 250 mg; thiamine, 500 mg; riboflavin, 450 mg; pyridoxine, 350 mg; cyanocobalamin, 0.8 mg; nicotinamide, 1700 mg; d-pantothenic acid, 750 mg; choline chloride, 35000 mg; $ZnCO_3$, 5700 mg; $MnSO_4$, 8250 mg; $FeSO_4$, 3890 mg; $CuSO_4$, 1160 mg; $CoSO_4$, 17 mg.

Table 2. **Particle size distribution of the experimental diets and their ingredients (%)**

Particle size class	experimental diet		ingredient	
	GC[1]	WPR[2]	Ground corn	Whole grain paddy rice
＞2.8 mm	0.28	56.12		86
2.0–2.8 mm	13.58	12.13	15.2	14
1.4–2.0 mm	35.39	6.65	42.1	
1.0–1.4 mm	17.06	5.18	18	
0.5–1.0 mm	13.14	7.06	10.3	
＜0.5 mm	20.65	13.2	14.4	

[1] The ground corn diet contained 75% corn.

[2] The whole grain paddy rice diet contained 9% corn and 65% whole grain paddy rice.

Table3. **Growth performance of broilers fed the ground corn and whole grain paddy rice diets during 14 to 28d of age (n=30)**

Item	GC[1]	WPR[2]
Feed intake (g)	1653	1861
Initial wegiht (g)	409±59	401±37
Final wegiht (g)	1445±203	1383±113

[1] The ground corn diet

[2] The whole grain paddy rice diet

Table 4. **Influence of the ground corn and whole grain paddy rice diets on the relative weight of the gizzard and pH of its contents in 28-day-old broilers (mean ± SD, n = 6)**

	Relative weight of gizzard[1] (g/100g of BW)	Gizzard contents pH[2]
Ground corn diet	1.83 ± 0.19^{a}	3.15 ± 0.23
Whole grain paddy rice diet	2.37 ± 0.14^{b}	2.96 ± 0.27

[a, b] Means on the same line with different superscripts are significantly different ($p < 0.01$).

[1] Empty gizzard weight/body weight.

[2] pH of gizzard contents diluted (1:2) with deionized water.

Table 5. **Quantitative (log 10 colony-forming units (cfu)/g of content) and qualitative determination of the *Campylobacter jejuni* concentration in the cecum of chickens fed either a whole grain paddy rice diet (WPR) or ground corn diet (GC) (n = 6)**

Sample	*C. jejuni* [1]	
	Quantification[2]	Qualitative[3]
GC		
1	4.0×10^5	+
2	4.0×10^3	+
3	3.2×10^4	+
4	ND	-
5	1.4×10^4	+
6	5.6×10^4	+
sum[4]		5/6[a]
WPR		
1	ND	-
2	ND	-
3	ND	-
4	ND	-
5	ND	-
6	ND	-
sum[4]		0/6[b]

ND: no detected colonies; +: one or more colonies; -: no colonies.

[1] Thirty-day-old broiler chickens were inoculated with 2×10^3 cfu of *Campylobacter jejuni,* and after 48 h, *Campylobacter* specimens in the cecum contents were cultured.

[2] Diluted cecum samples were plated onto modified charcoal cefoperazone desoxycholate agar (CCDA) plates and incubated for 48 h at 42°C under microaerophilic conditions.

[3] Cecum samples were cultured in Preston broth for 24 h at 42°C, and one loopful of broth was plated onto CCDA agar and incubated for 48 h at 42°C under microaerophilic conditions.

[4] Positive/total samples.

[a,b] $p < 0.01$.

Chapter 3 Effects of a Whole-grain Paddy Rice Diet on the pH Distribution in the Gizzard and Retention Time of Digesta in the Crop of Broiler Chicks

Abstract

We previously reported that a diet containing 65% paddy rice suppressed the colonization of *Campylobacter jejuni* in the cecum of broiler chicks, suggesting that this type of diet has positive effects on upper gastrointestinal tract function in broilers. To reveal the possible mechanisms involved in this antibacterial effect of the whole-grain paddy rice diet, we performed experiments comparing the digesta passage rate in the crop and gizzard, the development of gizzard, and the pH distribution in the gizzard between groups of chicks fed two different diets, such as ground corn and whole-grain paddy rice. During these experiments, we made the following observations: the chicks in the group fed the whole-grain paddy rice diet had more developed gizzards and significantly larger crop content than the chicks in the group fed the ground corn diet. The chicks fed the whole-grain paddy rice diet retained the digesta in the crop for much longer and had less variation in the pH values in the gizzard than those fed ground corn. On the basis of these observations, we concluded that the hardness of the rice hull in whole-grain paddy rice leads to a larger amount and longer retention of content in the crop, as well as the uniformity of the internal pH of the gizzard, by promoting gizzard activity. We speculate that the hardness of the rice hulls promoted the grinding activity of the gizzard, resulting in the long retention time of the digesta in the crop and uniformity of the internal pH of the gizzard, which may sterilize or suppress *Campylobacter* growth in the gastrointestinal tract of broiler chicks.

Introduction

Chickens have several natural barriers in their upper gastrointestinal tract that help prevent pathogenic bacterial infection (Moen *et al*., 2012). For example, in the crop and gizzard, the most dominant bacteria is *Lactobacillus* (Rehman *et al*., 2007), which exerts a bactericidal or bacteriostatic effect on invading pathogenic bacteria by producing organic acids and bacteriocins (Fuller, 1973; Jin *et al*., 1996). In addition, the gizzard maintains a low pH, which produces a bactericidal environment for many bacteria, including *Campylobacter*, via the secretion of hydrochloric acid from the glandular stomach. As the gizzard plays a role in promoting digestive activity in the lower part of the digestive tract by triturating feed particles, its activity is influenced by factors such as hardness and the type and proportion of dietary fiber in feed (Nir *et al*., 1994b); as a result, the gizzard pH decreases, and it takes longer for gizzard contents to pass to the lower part (González-Alvarado *et al*., 2007; Jiménez-Moreno *et al*., 2009a). For example, by adding insoluble dietary fibers such as oat hulls in the diet of chickens, the gizzard pH decrease and this reduces the frequency of *Campylobacter* and *Salmonella* infection or a small number of bacteria in the intestinal tract in young broilers (Bjerrum *et al*., 2005; Santos *et al*., 2008).

In our previous study (Nishii *et al*., 2015), we examined the inhibitory effect of a diet containing whole-grain paddy rice on the colonization of *C. jejuni* in the cecum of broiler chicks and reported that feeding a diet containing 65% Whole-grain paddy rice to broiler chicks from 2 weeks of age significantly inhibited *C. jejuni* colonization in the cecum of chicks inoculated with the bacteria at 4 weeks of age. We also observed that the mean ratio of gizzard weight to body weight of the chicks fed the whole-grain paddy rice diet was significantly higher than that of control chicks fed a ground corn diet.

Therefore, we speculated that feeding of whole-grain paddy rice to chicks caused the inhibition of *C. jejuni* colonization in the cecum of broiler chicks, to the same extent as that caused by the feeding of oat hulls, by the improvement in gizzard activity, the decrease in gizzard pH, and the longer retention of contents in the gizzard.

In the present study, we focused on comparing the pH distribution and internal pH in the gizzard, bacteriological trend of the crop contents, and digesta passage rate in the crop and gizzard. Finally, we discussed the possible mechanism of the inhibitory effect of the whole-grain paddy rice diet on *C. jejuni* colonization in the upper gastrointestinal tract of broiler chicks.

Materials and Methods

Animal care

All procedures involving animals and their care conformed to the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006).

Experiment 1

Chick rearing and feeding procedures were similar to those conducted in our previous study (Nishii *et al*., 2015). Eighty 1-day-old female broiler chicks (Chunky) were raised in a flock-rearing area that was approximately 2 m^2 in size and covered with sawdust in a windowless broiler house. The rearing area temperature was controlled at 34°C on the first day of rearing and was gradually lowered to 25°C until the chicks reached 14 days of age. After 15 days of age, the room temperature was kept between 20 and 25°C. The duration of light exposure was continuously controlled until 7 days of age, and after 8 days, it was maintained at 20 h per day. During the experiment, the feed

intake and body weight of the birds were measured weekly. The chicks were given a starter feed until 14 days of age (Table 1) containing ground corn (68.5% on a weight basis) crushed into particles of less than 1.4 mm in size.

On day 14, the chicks were allocated into three groups: ground corn diet (GC), whole-grain paddy rice diet (WPR), and brown rice diet (BR). The diets for each group are shown in Table 1. The ground corn was supplied by Yao Feed Co., Ltd. (Osaka, Japan), and the whole-grain paddy rice (Momiroman) and brown rice were obtained from Kyoto Prefecture Agriculture Experiment Station (Kameoka-Shi, Kyoto, Japan). The particle size distributions of the three diets were determined by the dry sieving method and are summarized in Table 2. The feedstuff did not contain any antimicrobials or coccidiostats. All chicks had free access to feed and water throughout all the experiments.

During the experiment, the birds' feed intake and body weight were measured weekly. At 28 and 42 days of age, eight chicks from each flock were randomly selected and weighed, killed by anesthesia overdose by intravenous injection (sodium pentobarbital, Somnopentyl; Kyoritsu Pharmacy, Tokyo, Japan), and then dissected. The contents of each segment in the digestive tract (crop, proventriculus, gizzard, duodenum, jejunum, ileum, and cecum) were removed onto a plastic plate under aseptic conditions and weighed. A portion of the content (0.1 g) was collected for bacterial culture, and the remaining portion was subjected to the following analyses. The content was added to two volumes of deionized water in a 50-ml centrifuge tube, and the suspension was mixed using a vortex mixer for 1 min. The pH of the diluted mixture was measured using a glass electrode pH meter (9615-10D; Horiba, Kyoto, Japan) twice for each mixture. The empty gizzard was then washed with water, dried with desiccant paper, and weighed.

The total viable bacterial counts of *Lactobacilli*, coliform bacteria, and lactose-negative enterobacteria in the contents of the crop, gizzard, ileum, and cecum were quantitatively detected. The contents of each segment were diluted 1:10 (w/v) in dilution anaerobic buffer solution [composition in 1000 ml of purified water: KH_2PO_4, 4.5 g; Na_2HPO_4, 6.0 g; L-cysteine hydrochloride, 0.5 g; Tween 80, 0.5 g; agar, 1 g (Mituoka, 1994)], and the first diluted suspension was serially diluted in 10-fold steps using the dilution anaerobic buffer solution. Subsequently, the suspensions were spread onto the following agar culture media: Lactobacillus selective LBS agar (BBL, Becton Dickinson and Company, Sparks, MD, USA) supplemented with 0.8% Lab-Lemco Powder (Oxoid Ltd, Basingstoke, Hampshire, England), 0.1% sodium acetate trihydrate, and 0.37% acetate (modified-LBS) for *Lactobacilli* and MacConkey agar (Oxoid Ltd, Basingstoke, Hampshire, England) for coliform bacteria and lactose-negative enterobacteria. Modified-LBS agar plates were anaerobically incubated at 37°C for 48 h, and all growing colonies were counted. MacConkey agar plates were aerobically incubated at 37°C for 24 h, and coliform bacteria and lactose-negative enterobacteria were counted as red and colorless colonies, respectively.

Experiment 2

One hundred 1-day-old female broiler chicks (Chunky) were raised in the same manner as that in Experiment 1. On day 14, the chicks were allocated into two groups: GC and WPR. At 28 days of age, 17 chicks from each group were randomly selected and weighed and were killed by the same method as that in Experiment 1 (anesthesia overdose by injection). The gizzard and proventriculus were removed from the chicks, and the removed gizzard was incised from the ventral side of the proventriculus along the median line using surgical scissors to expose the gizzard content. Immediately after

the incision was made, 10 portions of the content, approximately 0.5 g each, were gently removed from the exposed content using a small pair of tongs at the 10 positions shown in Figure 1. These portions were put on the measurement section of a pH meter (B712; Horiba, Kyoto, Japan), added to 0.5 ml of deionized water, gently mixed, and the pH was then measured within 10 min after removal. The sites of content sample collection depicted in Figure 1 are anatomically labeled as follows: ①and ⑩: the vicinity of the proventriculus (proximal site), ⑧and ⑨: near the pylorus leading to the duodenum (distal site), and ②–⑦: the others (middle site).

Experiment 3

At 29 days of age, 32 birds from each group (GC and WPR) were randomly selected and orally administered 1 ml of titanium dioxide-water solution (0.426 g/ml) (Wako Pure Chemical Industries, Osaka, Japan) using a 1-ml syringe attached to a flexible tube. Four birds from each group were killed by the same method as that in Experiment 1 (anesthesia overdose by intravenous injection) at 20, 40, 60, 80, 100, 120, 180, and 240 min after the administration of the titanium dioxide solution. Immediately after killing, the crop was removed from the birds killed at 20, 40, 60, 80, 100, and 120 min. The gizzard was also removed from the birds killed at 60, 120, 180, and 240 min. The contents of the crop and gizzard were transferred into 100-ml beakers and dried at 105°C overnight using a ventilation dryer. The dried content was crushed using a food mill and was then subjected to quantitative determination of titanium dioxide by the method by Short *et al*. (1996).

The mean retention time (MRT) of the digesta was estimated using the following calculation, where t (min) is the elapsed time after the administration of titanium dioxide to the chicks and $R_{crop}(t)$ and $R_{gizzard}(t)$ are the residual amounts of titanium

dioxide in the crop and gizzard, respectively, assuming that the residual amount of titanium dioxide in the crop at the time of administration of titanium dioxide (t = 0), $R_{crop}(0)$, is one.

$$dR_{crop}(t)/dt = -a\,R_{crop}(t)$$

$$dR_{gizzard}(t)/dt = a\,R_{crop}(t) - b\,R_{gizzard}(t)$$

where the small italic letters, a and b, are constants.

By solving these differential equations, the following equations are obtained.

$$R_{crop}(t) = e^{-at} \qquad (1) \text{ for the crop}$$

$$R_{gizzard}(t) = a/(a-b)(e^{-bt} - e^{-at}) \qquad (2) \text{ for the gizzard}$$

Finally, MRTs are calculated as follows (Dhanoa *et al.*, 1985):

$$\mathrm{MRT}_{crop} = 1/a \qquad (3) \text{ for the crop}$$

$$\mathrm{MRT}_{gizzard} = 1/b \qquad (4) \text{ for the gizzard}$$

The residual amounts of titanium dioxide in the crop and gizzard measured at each time point after its administration in the present experiment were substituted into equations (1) and (2), respectively, and the constants, a and b, were determined by the least squares method. These calculations were conducted using the solver function of Microsoft Excel 2010 (Redmond, WA, USA). MRTs in the crop and gizzard were calculated by equations (3) and (4), respectively.

Statistical analysis

In Experiment 1, the differences in the mean values of the chicks' body weight, relative weight of the gizzard, pH and weight of the content of the gizzard, and the bacterial counts in the contents of the crop, gizzard, ileum, and cecum between the chick groups were statistically analyzed by multiple comparisons using the Tukey–Kramer test. In Experiment 2, the differences in the pH values among the three sites

were analyzed using two-way analysis of variance (ANOVA) (the factors were the chicks' groups and measured sites). When a significant difference was observed in the dispersion component, the mean value of the component was subjected to a significant difference test using the Tukey–Kramer method. The coefficient of variations of the individual chicks was calculated, along with the mean and the standard deviation of the pH values at the 10 measurement points of the gizzard for each chick. Significant differences of the mean of the coefficient of variation were analyzed using ANOVA. *P*-values less than 0.05 were considered statistically significant. Both tests were conducted using Microsoft Excel 2010 add-in software.

Results

Experiment 1

Table 3 shows the comparison of the change in the mean body weight of the chicks in the three experimental groups in addition to their feed intake during Experiment 1. Although the feed intake was not significantly different between the three groups during the observation period, the mean body weight of the chicks in the BR group was significantly higher than that of the GC and WPR groups at 28 ($p = 0.021$) and 42 ($p = 0.041$) days of age. Table 4 shows the comparisons of the relative weights of the gizzard and pH values in the crop and gizzard among the three groups. Statistical analysis (Tukey–Kramer test) showed significant differences in the relative weight of the gizzard (gizzard weight/100 g of body weight) of the chicks among the three groups: at 28 ($p < 0.001$) and 42 ($p < 0.001$) days of age, the value of this parameter was significantly higher in the WPR group than in the other two groups. The order of the relative weights of the gizzard was WPR > GC > BR. There was no significant difference between any

pair of the mean pH values of the contents of the crop and gizzard at any age in the chicks.

Tables 5 and 6 show the weight of the content of the crop, proventriculus, gizzard, ileum, and cecum at 28 and 42 days of age, respectively. At 28 days of age, there was significantly more content in the crop in the WPR group than in the GC group (p = 0.004); in the lower gastrointestinal tract, there was significantly more content in the duodenum and ileum in the WPR group than in the BR and GC groups (p = 0.018). At 42 days of age, there was significantly more content in the crop in the WPR group than in the GC group (p = 0.001), and in the lower gastrointestinal tract, there was significantly more content in the ileum in the WPR group than in the GC group (p = 0.005).

Table 7 shows the populations of *Lactobacilli*, coliform bacteria, and lactose-negative enterobacteria in each segment at 28 and 42 days of age. At 28 days of age, the GC group had the lowest numbers of *Lactobacilli* in the gizzard among the three groups, and the number of *Lactobacilli* in the cecum of the GC group was significantly lower than that of the BR group (p = 0.006); further, there were no significant differences in the number of *Lactobacilli* in the crop or ileum among the three groups at either 28 or 42 days of age. The number of coliform bacteria did not significantly differ among the three groups, and neither was there any significant difference among the various segments or at any age, except for between the WPR and BR groups in the ileum at 28 days of age. Lactose-negative enterobacteria were not detected in the gizzard of chicks in the three groups at any age.

Experiment 2

Table 8 shows the comparison of the mean pH values in the three sites (proximal,

distal, and middle site) between the GC and WPR groups. In the WPR group, there were no significant differences in the pH values among the three sites; conversely, in the GC group, the pH value at the proximal site was significantly lower than that of the other two sites. To clarify the uniformity of the pH distribution in the gizzard, we calculated and compared the coefficient of variations of the pH values at the 10 measurement sites for each group. As shown in Table 8, the coefficient of variation for the WPR group was significantly smaller than that of the GC group (p = 0.026), which suggests that the variation of pH values in the gizzard of the chicks in the WPR group was less than that of the chicks in the GC group. In order to examine survival pH ranges of *Campylobacter*, we summarized the three pH range frequency in the gizzard in the two chick groups as Table 9. This table shows that the sampling sites with pH values lower than pH 3 and higher than pH 4 accounted for 29.1% and 15.0% (on average), respectively, of all the sampling points in each group (n = 170).

Experiment 3

Figures 2 and 3 show the amount of titanium dioxide detected in the crop and gizzard, respectively, after various lengths of time following the administration of titanium dioxide as a percentage of the initial amount of titanium dioxide. These figures also show the residual curves drawn using Equations (1) and (2). MRT was calculated based on these regression curves (Table 10). In the GC group, MRTs through the crop and gizzard were 41 and 34 min, respectively. In the WPR group, MRTs through the crop and gizzard were 115 and 21 min, respectively. Titanium dioxide was retained in the crop for longer in the WPR group than in the GC group, whereas there was no difference in MRT in the gizzard between the two groups.

Discussion

The gizzard plays a role in grinding coarse feed particles until a certain minimal critical size by performing its muscular activity, before its contents are passed into the lower digestive tract. Therefore, when chicks are fed feed materials of certain grinding resistance, they develop stronger gizzard function (muscular activity) than when fed regular chicken feed. These phenomena have been reported for diets containing oat hull (Jiménez-Moreno *et al*., 2009b; González-Alvarado *et al*., 2010; Mateos *et al*., 2012), coarsely crushed corn (Nir *et al*., 1994a; Nir *et al*., 1994b; Singh *et al*., 2014), and whole wheat (Engberg *et al*., 2004; Bjerrum *et al*., 2005; Gabriel *et al*., 2008). In the present experiment, the level of gizzard development was in the order of WPR > GC > BR (estimated by the relative weight of the gizzard; see Table 4). This observed order of gizzard development seems to be consistent with the frequency of resistance of grinding activity in the gizzard, when each feed material was soaked with water in the crop. This soft property of brown rice is confirmed by the fact that brown rice has a constant stiffness in the dry state, but absorbs water easily and softens when it is soaked for 15–30 min in water (Jagtap *et al*., 2008; Kong *et al*., 2011). In contrast to brown rice, paddy rice is composed of brown rice and hulls; the proportion of rice hulls is equivalent to approximately 20% by weight of paddy rice. Rice hulls are highly resistant to grinding in the upper digestive tract because they have a silica layer inside the backbone of the epidermis, which forms a hard, rough surface that imparts a specific texture. A previous study reported that gizzard development is superior in chicks fed a diet containing 2.5% or 5% rice hull than in those fed a diet without rice hulls (Mateos *et al*., 2012). In the present study, we used a whole-grain paddy rice diet containing 60% paddy rice (Table 1), which corresponds to a diet containing 12% rice hulls. Therefore, we presume that

the texture of the hulls in paddy rice enhanced the grinding activity of the gizzard, thus promoting gizzard development.

In Experiment 1, there was no difference in the pH of the gizzard contents among the three groups. However, in Experiment 2, we found variations in pH in the gizzard contents in the GC and WPR groups; the mean coefficients of variation of the 10 pH measurement sites in the individual chicks were 0.1079 and 0.0785 for the GC and WPR groups, respectively (Table 8). We interpreted the mechanism of these pH variations observed in the gizzard as follows. The pH at the proximal site (①⑩ in Fig. 1) was generally low because gastric juice from the proventriculus flows into this site. The higher pH at the distal site (Fig. 1⑧⑨) reflects the inflow of bicarbonate and bile from the duodenum associated with reverse peristalsis (Duke, 1992). In addition, calcium salts in the diet work as a buffer to raise the pH in the gizzard (Guinotte *et al*., 1995; Lawlor *et al*., 2005; Walk *et al*., 2012). The inflow and outflow of feed particles simultaneously occur in the gizzard, producing dynamic changes in the pH; as a result, the gizzard contents have various pH values.

Additionally, we found that the coefficient of variance of pH in the gizzard contents in the WPR group was significantly smaller than that in the GC group (Table 8). There was no significant difference in the pH values at the three sites in the WPR group, whereas in the GC group, the pH value at the proximal site was significantly lower than that at the other two sites (Table 8). These results may explain the fact that the orally ingested *Campylobacter* survived in the gizzard of the WPR group in our previous study (Nishii *et al*., 2015). The optimum pH of *Campylobacter* has been reported as pH 6.5–7.5 (Jackson *et al*., 2009). In *in vitro* experiments, Chaveerach *et al*. (2002) showed that the numbers of *Campylobacter* decreased at values lower than pH 4 and that *Campylobacter* is sterilized at pH values below 3. In the present study, the average pH

range of the gizzard was pH 3.3 to 3.4, and the number of *Campylobacter* was reduced under these conditions. While the average pH in the gizzard was low enough to prevent bacterial growth, 15% of the points for the measurement of pH in the gizzard had values higher than pH 4, i.e., the viable pH range for bacterial growth, which would enable *Campylobacter* to survive (Table 9). Under such conditions (the gizzard has points where *Campylobacter* may be able to survive), the promoted grinding activity of the gizzard could contribute to the elimination of these high pH areas in the gizzard through uniform pH distribution if the gizzard content was sufficiently mixed. The results of our study show that when broiler chicks are fed whole-grain paddy rice, the coefficient of variation of the gizzard pH is significantly smaller than that in chicks fed ground corn. Thus, we surmised that the hardness (grinding resistance of the food material) of the whole-grain paddy rice promoted the development of the activity of the gizzard and the uniformity of the internal pH of the gizzard, thus eliminating the areas of higher pH in the gizzard where *Campylobacter* can survive.

MRT of the gizzard contents was similar between the GC and WPR groups (Table 10). The grinding activity of the gizzard crushes coarse feed particles until a certain critical size is selectively retained ingested feed in the gizzard, before passage of the food to the lower gastrointestinal tract (Svihus, 2011). Therefore, in this experiment, MRT in the gizzard may have been influenced by the rheological properties due to their particle sizes of the marker used. A previous study used Cr-mordanted sunflower hulls and titanium dioxide as markers and showed that MRT of titanium oxide in the gizzard was significantly shorter than that of Cr-mordanted sunflower hulls (Rougière and Carré, 2010). We speculate that the insoluble fraction such as hulls of paddy rice in WPR retained in the gizzard for long time than the marker of titanium dioxide, because whole-grain paddy rice was resistant to milling as Rougière and Carré (2010) found that

sunflower hulls were retained in the gizzard.

In the present study, there was significantly more content in the crop in the WPR group than in the GC group (Tables 5 and 6). Thus, we discuss the possible causes for variations in the crop content and its inhibitory effect on *Campylobacter* below.

The crop is a storage organ that holds the ingested feed to be sent to the gizzard (Jackson and Duke, 1995). The storage of feed in the crop, therefore, is closely related to feed intake regulation, such as the frequency of feeding and the time spent feeding. The accumulation of feed in the crop (large crop content) has been reported in chickens subjected to time limitations for feeding, such as intermitting feeding, lighting schedule, and some feed types (Barash *et al*., 1993; Duve *et al*., 2011). In contrast, with *ad libitum* feeding, chickens eat small amounts approximately every half hour and do not tend to store ingested food in the crop (Svihus, 2014). In the present study, the chicks were raised under *ad libitum* feeding conditions, and the amount of crop content in the GC group (Tables 5 and 6) was small (6.6 ± 1.4 g and 4.2 ± 1.1 g at 28 and 42 days of age, respectively), which is in agreement with the findings reported by Svihus (2014). However, Tables 5 and 6 show that although the chicks in the WPR group were raised under *ad libitum* feeding conditions, they had significantly larger crop contents (16.0 ± 2.9 g and 30.2 ± 5.4 g at 28 and 42 days of age, respectively) than those of the GC group. This phenomenon could be explained by Savory's (1985) findings that chickens had the largest crop and gizzard contents when they were fed diets nutritionally "diluted" by adding 40% cellulose. In our experiments, the whole-grain paddy rice diet contained 15% less metabolizable energy than the ground corn diet (Table 1) because the hulls of paddy rice are not digestible. In other words, the whole-grain paddy rice diet supplied in this experiment could be considered as a diluted feed similar to that described by Savory (1985).

Also, it is likely that the decrease in the passage of digesta to the lower gastrointestinal tract results from the hulls in the whole-grain paddy rice diet, which are resistant to grinding, and they are thus retained in the gizzard for a long time (Vergara *et al*., 1989). It seems to become a limiting factor (regulator) of feed digestion of the entire gastrointestinal tract like as a time limit for feeding or food deprivation. We speculate that whole-grain paddy rice acts as a nutritionally "diluted" cereal and adds resistance to the grinding activity in the gizzard. Feeding whole-grain paddy rice to broiler chicks may result in the accumulation of large amounts of feed in the crop in order to avoid hunger, or in other words, to satisfy energy requirements by increasing the feed intake (Denbow, 1994; Ferket and Gernat, 2006; González-Alvarado *et al*., 2010).

This larger crop content likely led to the effect of "the larger the crop content, the longer the retention in the crop" observed in this study (in Experiment 3; Figure 2, Table 10). Table 10 shows that MRTs of the digesta in the crop were 41 and 115 min in the GC and WPR groups, respectively. We speculate that the longer retention time in the crop of the chicks in the WPR group played an important role in the suppression of *Campylobacter* in our previous report (Nishii *et al.*, 2015). The reason for this is that *Lactobacilli*, which are the dominant flora in the crop, suppress the survival of pathogenic bacteria that have entered via the oral route, depending on the contact time with *Lactobacilli*. Chaveerach *et al*. (2002) reported that when 10^3 CFU/g of *Campylobacter* were exposed to pH 4.5 or pH 5.0 water-diluted acetic acid, the number of bacteria decreased over time and were not detected after 2 or 8 h, respectively. In the present study, there was no significant difference in the number of *Lactobacilli* or in the pH of the crop content between the GC and WPR groups; however, the pH range in the crop was low enough to inhibit bacterial growth. Therefore, when the crop is invaded by *Campylobacter*, it is possible that the longer retention of the content in the crop in

chicks fed whole-grain paddy rice may have a sterilizing or bacteriostatic effect on the *Campylobacter*. Thus, we surmised that the larger amount and longer retention of the crop content in the WPR group may exert a bacteriostatic effect due to the longer co-existence of the pathogenic bacteria with *Lactobacilli*, which exhibits bactericidal and bacteriostatic actions, due to the low pH in the crop.

Although the different diets had no effect on the pH range in the gizzard, bacterial growth was inhibited as the gizzard maintains a lower pH environment than that in the crop, which is supported by our observation that the populations of *Lactobacilli*, coliforms, and lactose-negative enterobacteria in the gizzard were significantly smaller than those in the crop (Table 7). The amount of *Lactobacilli* in the gizzard of the chicks in the WPR group, BR was significantly higher than that of GC, it is not obvious why the populations of *Lactobacilli* in the gizzard were different between three diets.

In conclusion, we speculate that there are two reasons why a whole-grain paddy rice diet inhibits *C. jejuni* colonization in the cecum of broiler chicks. First, the hardness (grinding resistance of the food material) of the whole-grain paddy rice promoted the development of gizzard activity; thus, this stronger grinding activity of the gizzard eliminated the areas of higher pH in the gizzard where *Campylobacter* could survive. Second, the larger amount and longer retention of the content in the crop in the WPR group had a bacteriostatic effect due to the longer co-existence of the pathogenic bacteria with *Lactobacilli*, which exhibit bactericidal or bacteriostatic action, due to the low pH in the crop.

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Table 1. **Comparison of the experimental diets**

Ingredients (%)	Starter diet[1] (1 to 14 days)	Grower diet (15 to 42 days)		
		Ground corn diet	Whole-grain paddy rice diet	Brown rice diet
Ground corn	65.0	70.0	10.0	10.0
Paddy rice	—	—	60.0	—
Dehulled rice	—	—	—	60.0
Soybean meal	20.0	20.0	20.0	20.0
Fish meal (65% crude protein)	7.0	5.0	5.0	5.0
Corn gluten meal	2.0	—	—	—
Soybean oil	3.0	2.0	2.0	2.0
Calcium carbonate	0.5	0.5	0.5	0.5
Tricalcium phosphate	0.6	0.6	0.6	0.6
Dicalcium phosphate	0.5	0.5	0.5	0.5
Manganese sulfate	0.015	0.015	0.015	0.015
Sodium chloride	0.25	0.15	0.19	0.19
DL-methionine	0.3	0.25	0.25	0.25
L-lysine HCl	0.5	0.4	0.4	0.4
Riboflavin	0.0004	0.00025	0.00025	0.00025
Copper sulfate	0.0005	0.001	0.001	0.001
Zinc sulfate	0.0005	0.004	0.004	0.004
Folacin	0.00004	0.00003	0.00003	0.00003
L-threonine	0.25	0.25	0.25	0.25
Choline chloride	0.05	—	—	—
Calcium pantothenate	0.0004	—	—	—
Nicotinamide	0.003	—	—	—
Vitamin/mineral premix[2]	0.3	0.3	0.3	0.3
Calculated				
Crude protein (%)	21.1	18.2	17.5	18.1
Metabolizable energy (kcal/kg)	3,056	3,120	2,748	3,120

[1] The starter diet was ground and then passed through a sieve with a mesh size of 1.4 mm.

[2] Vitamin and mineral premix including the following(per kg of the diet): retinol (retinyl acetate), 3,500,000 IU; cholecalciferol, 700,000 IU; vitamin E (DL-α-tocopheryl acetate), 600 mg; menadione, 250 mg; thiamine, 500 mg; riboflavin, 450 mg; pyridoxine, 350 mg; cyanocobalamin, 0.8 mg; nicotinamide, 1,700 mg; D-pantothenic acid, 750 mg; choline chloride, 35,000 mg; $ZnCO_3$, 5,700 mg; $MnSO_4$, 8,250 mg; $FeSO_4$, 3,890 mg; $CuSO_4$, 1,160 mg; $CoSO_4$, 17 mg.

Table 2. **Particle size distribution of the grower diet (supplied from 15 to 42 days of age)**

Particle size class	Ground corn diet[1]	Whole-grain paddy rice diet[2]	Brown rice diet[3]
>2. 8 mm	0.54	5.58	0.54
2.0–2.8 mm	14.80	60.64	5.68
1.4–2.0 mm	36.61	11.35	69.97
1.0–1.4 mm	17.08	6.28	7.66
0.5–1.0 mm	11.90	5.74	5.74
<0.5 mm	19.08	10.42	10.42

[1] The ground corn diet contained 70% corn.

[2] The whole-grain paddy rice diet contained 10% corn and and 60% paddy rice.

[3] The brown rice diet contained 10% corn and and 60% dehulled rice.

Table 3. **Mean body weights and feed intake in the three broiler chick groups from 14 to 42 days of age**

	n	Group			*P* -value
		GC[1]	WPR[2]	BR[3]	
Body weight (g)					
14 days	19	343 ± 8	339 ± 10	356 ± 5	0.323
28 days	19	1388 ± 21[b]	1389 ± 31[b]	1484 ± 28[a]	0.021
42 days	11	2542 ± 38[ab]	2514 ± 55[b]	2732 ± 89[a]	0.046
Feed (g/bird)					
14 to 21 days		662	648	687	
21 to 28 days		628	677	657	
28 to 35 days		621	646	655	
35 to 42 days		990	974	977	

The body weight values are presented as the mean ± standard error of the mean.

[a-b] Means within the same row with different superscripts are significantly different ($p < 0.05$).

[1] The ground corn diet.

[2] The whole-grain paddy rice diet.

[3] The brown rice diet.

Table 4. **The mean pH values in the gizzard and crop and the relative weight of the gizzard contents in the three chicks' groups**

Group	pH[1]				Relative weight of gizzard (g/100 g body weight)	
	Crop		Gizzard			
	28 days	42 days	28 days	42 days	28 days	42 days
GC[2]	5.08 ± 0.14	4.71 ± 0.19	3.51 ± 0.13	3.84 ± 0.19	1.76 ± 0.09[b]	1.43 ± 0.05[b]
WPR[3]	5.00 ± 0.12	5.04 ± 0.01	3.88 ± 0.11	4.01 ± 0.17	2.13 ± 0.03[a]	1.74 ± 0.06[a]
BR[4]	4.83 ± 0.04	4.75 ± 0.10	3.83 ± 0.14	3.92 ± 0.13	1.47 ± 0.04[c]	1.17 ± 0.03[c]
	P -value					
Group	0.285	0.188	0.098	0.751	< 0.001	< 0.001

The data are presented as the mean ± standard error of the mean, n = 8.

[a-c] Means within the same column with different superscripts are significantly different ($p < 0.05$).

[1] pH of gizzard contents diluted (1:2) with deionized water.

[2] The ground corn diet.

[3] The whole-grain paddy rice diet.

[4] The brown rice diet.

Table 5. **Weight of the contents in different parts of the intestine at 28 days of age**

Items (g)	Group			*P* -value
	GC[1]	WPR[2]	BR[3]	
Crop	6.6 ± 1.4[b]	16.0 ± 2.9[a]	13.1 ± 1.6[ab]	0.014
Proventriculus	1.6 ± 0.1	1.5 ± 0.1	1.2 ± 0.2	0.235
Gizzard	12.5 ± 0.8	14.5 ± 0.6	13.8 ± 0.8	0.190
Duodenum	5.7 ± 0.2[ab]	6.7 ± 0.5[a]	5.0 ± 0.4[b]	0.018
Jejunum	20.3 ± 1.5	24.8 ± 1.4	20.6 ± 1.5	0.071
Ileum	16.5 ± 0.8[b]	19.5 ± 0.7[a]	14.9 ± 0.6[ab]	<0.001
Cecum	9.2 ± 1.2	6.8 ± 1.0	6.6 ± 0.7	0.137

The data are presented as the mean ± standard error of the mean, n = 8.

[a-b] Means within the same row with different superscripts are significantly different ($p < 0.05$).

[1] The ground corn diet.

[2] The whole-grain paddy rice diet.

[3] The brown rice diet.

Table 6. **Weight of the contents in different parts of the intestine at 42 days of age**

Items (g)	Group			*P* -value
	GC[1]	WPR[2]	BR[3]	
Crop	4.2 ± 1.1[b]	30.2 ± 5.4[a]	16.3 ± 5.0[ab]	0.001
Proventriculus	1.6 ± 0.2[a]	0.7 ± 0.1[b]	1.9 ± 0.3[a]	0.001
Gizzard	16.3 ± 0.9	20.9 ± 1.1	18.2 ± 1.8	0.077
Duodenum	6.5 ± 0.5	7.4 ± 0.7	7.1 ± 0.9	0.631
Jejunum	33.1 ± 1.5	33.3 ± 1.5	29.3 ± 1.1	0.085
Ileum	18.2 ± 0.4[b]	24.5 ± 1.7[a]	20.9 ± 1.1[ab]	0.005
Cecum	9.8 ± 1.4	6.3 ± 1.1	9.5 ± 1.2	0.123

The data are presented as the mean ± standard error of the mean, n = 8.

[a-b] Means within the same row with different superscripts are significantly different ($p < 0.05$).

[1] The ground corn diet.

[2] The whole-grain paddy rice diet.

[3] The brown rice diet.

Table 7. **Bacterial counts (log10 cfu/g) in the contents of different parts of the intestine (n = 8)**

Group	GC[1]		WPR[2]		BR[3]		*P* -value group	
Time (day)	28	42	28	42	28	42	28	42
Lactobacilli								
Crop	8.36 ± 0.12	9.36 ± 0.19	8.41 ± 0.24	9.20 ± 0.10	8.69 ± 0.16	9.05 ± 0.20	0.459	0.437
	(8/8)[4]	(8/8)	(8/8)	(8/8)	(8/8)	(8/8)		
Gizzard	5.62 ± 0.30^{b}	5.03 ± 0.65^{ab}	6.49 ± 0.10^{a}	6.34 ± 0.30^{ab}	6.63 ± 0.08^{a}	6.37 ± 0.27^{ab}	0.002	0.069
	(8/8)	(8/8)	(8/8)	(8/8)	(8/8)	(8/8)		
Ileum	8.52 ± 0.15	7.89 ± 0.42	8.49 ± 0.16	7.86 ± 0.20	8.64 ± 0.11	8.06 ± 0.17	0.739	0.692
	(8/8)	(8/8)	(8/8)	(8/8)	(8/8)	(8/8)		
Cecum	8.27 ± 0.21^{b}	8.83 ± 0.40^{ab}	8.66 ± 0.08^{ab}	9.22 ± 0.21^{ab}	8.99 ± 0.07^{a}	9.35 ± 0.33^{ab}	0.006	0.500
	(8/8)	(8/8)	(8/8)	(8/8)	(8/8)	(8/8)		
Coliform bacteria								
Crop	4.87 ± 0.38	3.26 ± 0.55	4.77 ± 0.25	3.43 ± 0.39	4.11 ± 0.21	3.36 ± 0.52	0.160	0.965
	(8/8)	(8/8)	(8/8)	(8/8)	(8/8)	(8/8)		
Gizzard	2.35±0.27	2.90 ± 0.52	3.67 ± 0.71	2.81 ± 0.53	2.95	1.78		
	(2/8)	(5/8)	(6/8)	(5/8)	(1/8)	(1/8)		
Ileum	6.15 ± 0.27^{ab}	5.88 ± 0.28^{ab}	6.49 ± 0.23^{a}	5.14 ± 0.35^{ab}	5.20 ± 0.32^{b}	5.40 ± 0.37^{ab}	0.009	0.298
	(8/8)	(8/8)	(8/8)	(8/8)	(8/8)	(8/8)		
Cecum	7.36 ± 0.29	7.28 ± 0.22	7.59 ± 0.27	7.16 ± 0.32	6.94 ± 0.29	6.99 ± 0.30	0.285	0.764
	(8/8)	(8/8)	(8/8)	(8/8)	(8/8)	(8/8)		
Lactose-negative enterobacteria								
Crop	4.12 ± 0.51	ND[5]	4.02 ± 0.24	2.15 ± 0.15	3.17 ± 0.37	2.21 ± 0.43		
	(5/8)	(0/8)	(2/8)	(4/8)	(5/8)	(3/8)		
Gizzard	ND	ND	ND	ND	ND	ND		
	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)		
Ileum	3.48	ND	ND	ND	2.78	ND		
	(1/8)	(0/8)	(0/8)	(0/8)	(1/8)	(0/8)		
Cecum	5.95 ± 0.18	ND	6.48 ± 0.13	ND	6.14 ± 0.17	6.27 ± 0.20	0.099	
	(4/8)	(0/8)	(5/8)	(0/8)	(3/8)	(4/8)		

Values are colony-forming units per gram of the content (Mean ± SEM)

[a-b] Means within a line with different superscripts are significantly different ($p < 0.05$).

[1] The ground corn diet.

[2] The whole-grain paddy rice diet.

[3] The brown rice diet.

[4] Colonies detected on the plate/total number of colonies on the plate.

[5] ND = not detectable.

Table 8. **Comparision of the mean pH values at three different sites in the gizzard between the two groups of chicks**

Group	pH values at three different sites			*P* -value	Coefficient of variance[6]
	Proximal[3]	Middle[4]	Distal[5]		
GC[1]	3.06 ± 0.52[b] n = 34	3.33 ± 0.53[a] n = 102	3.58 ± 0.55[a] n = 34	<0.001	0.1079 ± 0.0402[x] n = 17
WPR[2]	3.23 ± 0.43 n = 34	3.41 ± 0.56 n = 102	3.47 ± 0.49 n = 34	0.136	0.0785 ± 0.0293[y] n = 17

The data are presented as the mean ± standard error of the mean.

[1] The ground corn diet.

[2] The whole-grain paddy rice diet.

[3] Contents at the collected site ① and ⑩ in Figure 1.

[4] Contents at the collected site ②, ③, ④, ⑤, ⑥, and ⑦ in Figure 1.

[5] Contents at the collected site ⑧ and ⑨ in Figure 1.

[6] The coefficient of variance was calculated on the basis of 10 sites in the gizzard of individual chicks.

[a,b] Means within the same row with different superscripts are significantly different ($p < 0.05$).

[x,y] Means within the same column with different superscripts are significantly different ($p < 0.05$).

Table 9. **Relative frequency of three classes of pH values at different sites in the gizzard**

Group	Relative frequency (%)		
	pH < 3	pH 3–4	pH > 4
GC[1]	31.8	54.7	13.5
WPR[2]	26.5	57.1	16.5
Mean	29.1	55.9	15.0

[1] The ground corn diet.

[2] The whole-grain paddy rice diet.

Table 10. **Estimates of the mean retention time of titanium dioxide in the crop and gizzard**

Group	Mean retention time (min)	
	Crop	Gizzard
GC[1]	41	34
WPR[2]	115	21

[1] The ground corn diet.

[2] The whole-grain paddy rice diet.

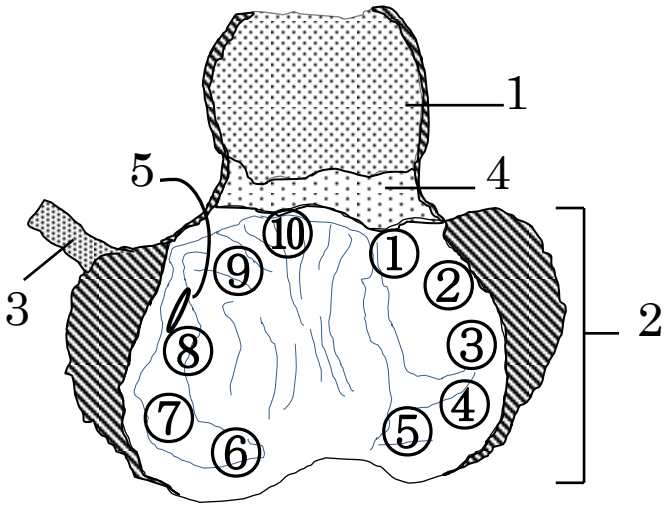

Fig. 1. **Distribution of points and position for the measurement of pH in gizzard contents.** The slanted line shows the ventral incision of the gizzard and proventriculus. 1, proventriculus; 2, gizzard; 3, proximal duodenum; 4, isthmus; 5, pylorus.

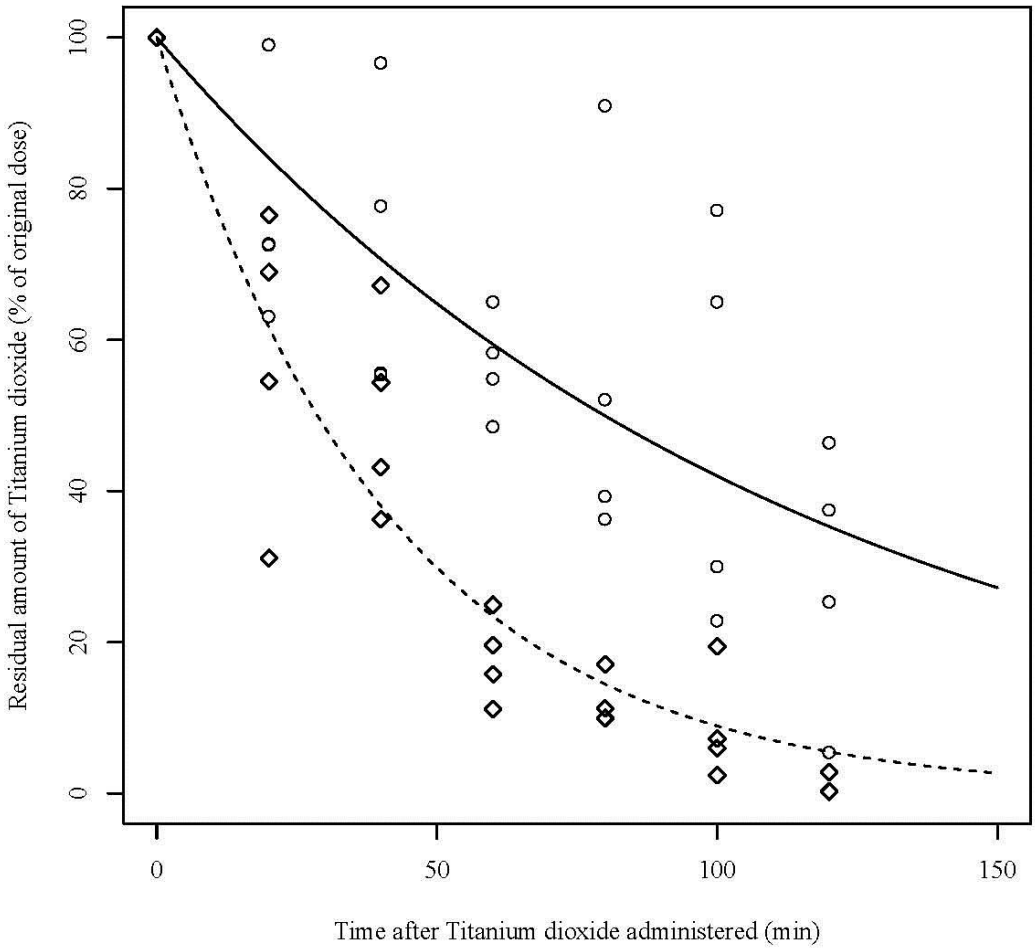

Fig. 2. **The amount of titanium dioxide detected in the crop at various time points after its administration (percentage of the admitted dose) (n = 4).** ○: WPR, ◇: GC. The residual curve calculated from Equation (1). Solid line: WPR; $R_{crop}(t) = 100e^{-0.0087t}$ ($r^2 = 0.49$), dashed line: GC; $R_{crop}(t) = 100e^{-0.0242t}$ ($r^2 = 0.77$).

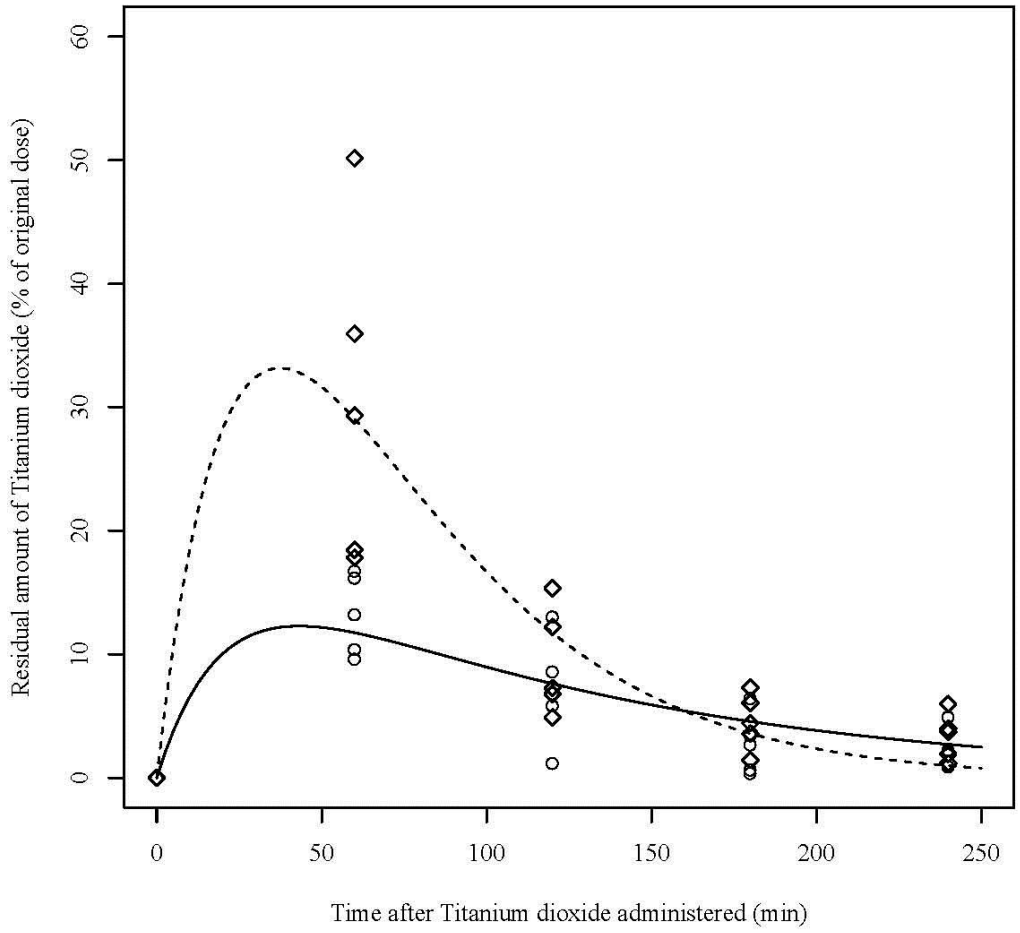

Fig. 3. **The amount of titanium dioxide detected in the gizzard at various time points after its administration (percentage of the admitted dose) (n = 4).** ○: WPR, ◇: GC. The residual curve calculated from Equation (2). Solid line: WPR; $R_{gizzard}(t) = -21.75(e^{-0.0486t} - e^{-0.0087t})$ ($r^2 = 0.48$), dashed line: GC; $R_{gizzard}(t) = -450.47(e^{-0.0296t} - e^{-0.0242t})$ ($r^2 = 0.33$).

Chapter 4 Summary

Chickens and other birds have specialized digestive organs called the crop and the gizzard that offer defense functions against oral invasion by pathogenic bacteria. As insoluble dietary fiber in feed has been proposed to enhance those defense functions in the upper intestinal tracts of chickens, the author decided to examine the inhibitory effect of a diet containing whole-grain paddy rice on the colonization of *C. jejuni* in the cecum of broiler chicks. The results showed that feeding a diet containing 65% whole-grain paddy rice to broiler chicks from 2 weeks of age significantly inhibited *C. jejuni* colonization in the cecum of chicks that had been inoculated with the bacteria at 4 weeks of age (Chapter 2).

To reveal the possible mechanisms involved in this antibacterial effect of the whole-grain paddy rice diet, experiments were performed to compare the digesta passage rate in the crop and gizzard, the development of gizzards, and the pH distribution in the gizzard between groups of chicks fed different diets. During these experiments, it was observed that the chicks in the group fed the whole-grain paddy rice diet had more developed gizzards and significantly larger crop content than the control chicks fed the ground-corn diet. Chicks consuming the whole-grain paddy rice diet retained the digesta in the crop for much longer times and had less variation in the gizzard pH values than did chicks fed ground corn. On the basis of these observations, the author speculates that the hardness of the rice hulls promoted the grinding activity of the gizzard, resulting in the long retention time of larger amounts of digesta in the crop and uniformity of the internal pH of the gizzard, with the possible result that may kill *C. jejuni* organisms or at least suppress their growth in the gastrointestinal tract of broiler chicks (Chapter 3).

The results obtained in this study indicate that feeding of whole-grain paddy rice induced both physiological and histological changes in the crop and the gizzard, and that those changes possibly suppressed the colonization of *Campylobacter* in the ceca of broiler chickens. However, further studies will be needed to prove the suppressive and inhibitory effect of those changes on *Campylobacter* colonization.

The present results show that feed rich in insoluble dietary fiber could enhance the natural defense functions of chickens against pathogenic bacteria and suggests that this measure could be applied to protect chickens from opportunistic infection with other organisms such as *Escherichia coli* and *Staphylococcus*. Because those infections are serious problems at broiler production sites and poultry processing plants, the author would like to continue to investigate the effectiveness of feed rich in insoluble dietary fiber on the prevention of pathogenic bacterial infection. Through these investigations, the author would like to contribute to the establishment of better methods for safe production of broiler chickens for the market.

Chapter 5 Concluding Remarks

Several recent studies have revealed that dietary fiber found in feedstuff coarse particles such as whole wheat, coarsely ground corn, whole triticale, and whole-grain paddy rice have marked physiological effects on chickens' digestive tracts in addition to the enhancement of natural barriers existing in the upper gastrointestinal tract and the inhibitory effect on pathogenic bacteria. This thesis reports empirical evidence to support the above findings in a study using whole-grain paddy rice as feedstuff. For example, feeding of dietary insoluble fiber prompts grinding activity of gizzard and frequency of the gastroduodenal reflex, resulting in retention of the digesta in the upper gastrointestinal tract for an extended period of time. This retention in turn leads to longer activity of the digestive enzymes amylase and phytase, and acceleration of gastric juice and protease secretion in the proventriculus. Then it enhances digestive efficiency of starches, amino acids, and fat is improved (Svihus, 2011). Because improving the utilization efficiency of feed is very important for industrial poultry farms, it is expected that paddy rice can advance achievement of this goal.

Japanese agriculture can potentially produce several million tons of rice per year, because Japan has 2.46 million ha of paddy rice farmland (Ministry of Agriculture Forestry and Fisheries statistics). However, at present, actual rice-planted farmland has remained at the level of 64% (1.58 million ha) as a consequence of the decrease in market demand for rice. If idle farmlands are revived and converted to paddy fields to produce rice for feed to be used by livestock farmers, not only can stable feed prices be achieved, management of livestock farms can also be improved. Additional positive effects on the local community would include national land conservation.

The author would like to continue to study the best means of feeding paddy rice to

broilers, so as to contribute to the sustainability and development of local communities.

References in Chapter 5

Svihus B. The gizzard: function, influence of diet structure and effects on nutrient availability. World's Poultry Science journal, 67: 207-224. 2011.

List of publications

Inhibitory Effect of Whole Grain Paddy Rice Feeding on the Colonization on the Colonization of *Campylobacter jejuni* in the Cecum of Broiler Chicks

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Effects of a Whole-grain Paddy Rice Diet on the pH Distribution in the Gizzard and Retention Time of Digesta in the Crop of Broiler Chicks

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The author expresses a willingness to promote the development and dissemination of the research.

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