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Bitter Taste Receptor Antagonists Inhibit the Bitter taste of Canola Meal Extract in Chickens

Fuminori Kawabata*, Bapon Dey †, Yuta Yoshida, Shotaro Nishimura and Shoji Tabata

Laboratory of Functional Anatomy, Faculty of Agriculture, Kyushu University,
744 Motooka, Nishi-ku, Fukuoka, 819−0395, Japan

Canola meal (CM) is a commonly used feedstuff; however, it is known to be bitter, and chickens have a low preference for it. The purpose of this study was to seek clarity regarding the taste quality of CM and find methods to increase the preference for CM by chickens. We examined whether CM activates the bitter taste receptors in chickens, whether chickens show aversive responses to CM, and whether an antagonist for bitter taste receptors inhibits the bitterness of CM. Using the Ca^{2+} imaging technique, we showed that CM contains bitter compounds, which activate the bitter taste receptors in chickens. Further, we showed that 6-methoxyflavanone (6-meth), an antagonist for the bitter taste receptors in chickens, inhibits the activation of these receptors by CM extract. Although chickens showed a low preference for the solution of the CM extract, their preference was improved by adding 6-meth in behavioral tests. These results suggest that the preference for CM could be improved by inhibiting the bitter taste receptors in chickens.

Key words: bitter taste receptor, canola meal, chicken

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Introduction

Canola meal (CM) is commonly used a chicken feed. However, the inclusion of a high level of CM in the diet decreases feed intake and body weight gain in broiler chickens (Khajali *et al*., 2011). It has been considered that these unfavorable effects are induced by glucosinolates, sinapine, tannin, and phytate present in CM (Khajali and Slominski, 2012). Alternatively, tannin, sinapine, and glucosinolates impart a bitter taste (Ismail *et al*., 1981; Soares *et al*., 2013; Wieczorek *et al*., 2018). We considered that the reduction in the intake of CM feed by chickens could be partly attributed to the bitterness of CM. Canola meal has a nutritionally suitable amino acid combination and is a useful feedstuff. Thus, it could be advantageous to increase the use of CM in the poultry industry by inhibiting the bitterness of CM and prevent the reduction in feed intake by chickens.

Many recent reports have mentioned that chickens sense bitter tastes via bitter taste receptors (Behrens *et al*., 2014; Cheled-Shoval *et al*., 2015, 2017; Hirose *et al*., 2015; Dey *et al*., 2017, 2018; Yoshida *et al*., 2019). Although chickens have three bitter taste receptor genes T2R1 (cT2R1), T2R2 (cT2R2), and T2R7 (cT2R7) (Go, 2006), two functional receptors cT2R1 and cT2R7 play key roles in detecting bitterness in their oral cavity (Hirose *et al*., 2015; Dey *et al*., 2017). It has been reported that chickens do not show a definite aversion to the solutions of cT2R2 agonists in behavioral tests (Dey *et al*., 2017), although cT2R2-expressing cultured cells were activated by the same bitter compounds, as determined by Ca^{2+} imaging (Behrens *et al*., 2014). Further, we have shown that 6-methoxyflavanone (6-meth), one of the polyphenols that functions as an antagonist for both cT2R1 and cT2R7 inhibited the aversive responses of chickens to bitter solutions in behavioral tests (Dey *et al*., 2017). Thus, 6-meth could help improve the intake of bitter feedstuffs such as CM by chickens.

In this study, we first examined whether an extract of CM activated the functional bitter taste receptors such as cT2R1 and cT2R7 through Ca^{2+} imaging studies. In addition, we confirmed that CM contains compounds that are bitter to

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Correspondence: Dr. Fuminori Kawabata, Physiology of Domestic Animals, Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyo-cho, Hirosaki, Aomori 036−8561, Japan.
(E-mail: kawabata@hirosaki-u.ac.jp)
* Present address: Physiology of Domestic Animals, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki, Japan.
† Present address: Department of Poultry Science, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh.

chickens. Second, we showed that 6-meth could partially inhibit the bitterness of CM at both the receptor and behavioral levels.

Materials and Methods

Animals

Rhode Island Red chicks of 4 weeks of age were used for this research. The study was carried out according to the Guide for Animal Experiments issued by Kyushu University, the Law Concerning the Human Care and Control of Animals (Law No. 105; October 1, 1973), the Act on the Welfare and Management of Animals (Law No. 105, 1973, the Ministry of Environment) and the Japanese Government Notification on the Feeding and Safekeeping of Animals (Notification No. 6; March 27, 1980). The animal experiments in this study were approved by Animal Experiments Review Board of Kyushu University (Approval number: A28-151-1).

Chemicals

Chemicals like 6-meth, dimethyl sulfoxide (DMSO), and adenosine triphosphate (ATP) were purchased from the Tokyo Chemical Industry (Tokyo), FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), and Sigma-Aldrich Japan (Tokyo), respectively. The CM was provided by J-Oil Mills Inc., Tokyo. We made a 100 mM 6-meth stock solution diluted with DMSO and a 10 mM ATP stock solution diluted with distilled water; both were stored at −20℃.

Preparation of CM Extract

Five grams of CM and 20 m*l* DMSO were added into a 50 m*l* tube. The mixture was mixed for 30 min in a shaker and vortexed several times. Further, the mixture was filtrated using a filter paper and about 6 to 7 m*l* of clear liquid was collected, i.e., the CM extract. This CM extract was stored at 4℃. For Ca^{2+} imaging, we prepared working solutions diluted with a standard bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose at pH 7.4, adjusted with NaOH just before each experiment. For behavioral experiments, CM extract solutions were diluted with normal tap water before each experiment. All working solutions were prepared just before each experiment.

Behavioral Test

One-bowl drinking tests were conducted based on our previous report (Dey *et al*., 2017). Briefly, the behavioral test took place over 12 consecutive days with the first six days as a training period for the chicks and days 7 to 12 as an experimental period. The chicks were kept together for the first two days and then separated into individual pens. Commercial layer feed (Powerlayer 17Y; JA Kitakyushu Kumiai Shiryo, Fukuoka, Japan) was fed to the chicks *ad libitum* throughout the whole experiment. The chicks were supplied normal tap water for 24 h only on the first day; the drinking time was then restricted to only 10 min in each 24 h period beginning at 17:00 to train them to drink for a short period of time. Water and test solutions (CM extract solution or CM extract solution with 6-meth) were given on a randomized basis over the experimental period. To compensate for the evaporation loss from the bowl in the 10 min of exposure control tap water was set in a brooder box, and the amount of evaporation was subtracted from the volume of water or test solution intake.

Constructs

From our previous research, we took the cT2R1/pDisplay and cT2R7/pDisplay (Hirose *et al*., 2015; Dey *et al*., 2017) and amplified them for this experiment. The chimeric G-protein, $G\alpha_{16/gust44}$/pcDNA3.1(+) (Ueda *et al*., 2003), was gifted by Dr. Takashi Ueda (Nagoya City University).

Cell Culture

Human embryonic kidney (HEK)-derived 293T (HEK 293T) cells were maintained in Dulbecco's Eagle's medium (DMEM high glucose; FUJIFILM Wako Pure Chemical Corporation) containing 10% fetal bovine serum (GE Healthcare, Buckinghamshire, UK) and penicillin-streptomycin solution (×100) (FUJIFILM Wako Pure Chemical Corporation) at 37℃ and 5% CO_2.

Measurement of the Index of Cytosolic Ca^{2+} Concentrations

For the Ca^{2+} imaging experiments (Dey *et al*., 2017) HEK293T cells were transfected with either empty vector pDisplay for mock cells or the cotransfection of $G\alpha_{16/gust44}$/pcDNA3.1(+) with cT2R1/pDisplay or cT2R7/pDisplay using ScreenFect^{TM}A (FUJIFILM Wako Pure Chemical Corporation) on coverslips coated with poly-D-lysine (0.1 mg/mL; FUJIFILM Wako Pure Chemical Corporation). After transfection, the cells were incubated for 48 h at 37℃ and 5% CO_2. Further, the cells were loaded with 1.25 mM Fluo 4-AM solution for 30 min at 37℃ and 5% CO_2 in the dark. Fluo 4-AM solution was prepared according to the instructions of the manufacturer (Dojindo Laboratories, Kumamoto, Japan).

The coverslips were washed with the standard bath solution; fluo-4 fluorescence was measured in the standard bath solution using a confocal laser scanning microscope (Nikon A1R; Nikon Co., Tokyo, Japan). The coverslips were mounted in a chamber connected to a gravity flow system to deliver various stimuli. Chemical stimulation was applied by running a standard bath solution containing various reagents. Cell viability was confirmed by responses to 10 μM ATP.

Statistical Analysis

The data were expressed as means±SE. Statistical analyses were conducted using the paired *t*-test; differences with *p*- values <0.05 were considered to be significant.

Results

CM Extract Increased the Index of Cytosolic Ca^{2+} through cT2R1 and cT2R7

Stimuli of 0.001% and 0.01% CM extract solutions increased the relative fluorescence units (RFU) (the index of cytosolic Ca^{2+}) in both cT2R1-expressing cells and cT2R7-expressing cells (Fig. 1A and 1B). The mock (empty vector) cells were not affected by the 0.001% and 0.01% CM extract solutions (Fig. 1C). These results suggest that the CM extract contains agonists of both cT2R1 and cT2R7.

Behavioral Analyses Toward CM Extract in Chickens

As the CM extract contains agonists of cT2R1 and cT2R7,

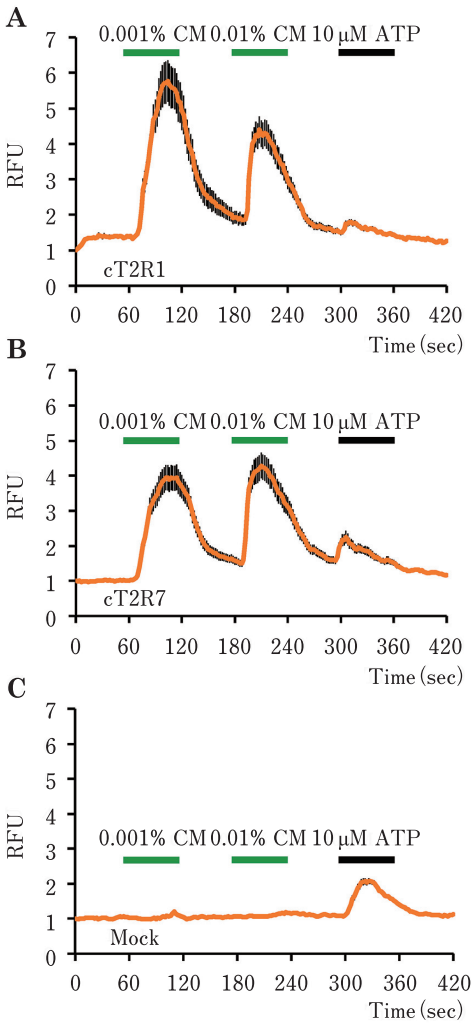

Fig. 1. **Canola meal (CM) extract increased the index of cytosolic Ca^{2+} through cT2R1 and cT2R7.** The representative data of the ratios of relative fluorescence units (RFUs), the index of cytosolic Ca^{2+} to the baseline value after stimulus with 0.001% CM extract, 0.01% CM extract, and 10 μM ATP in (A) cT2R1/pDisplay- and $G\alpha_{16/gust44}$/pcDNA3.1(+)-expressing cells, (B) cT2R7/pDisplay- and $G\alpha_{16/gust44}$ / pcDNA3.1 (+) -expressing cells, and (C) mock (empty vector) cells. Data are the means±SE of 17–29 cells.

we examined whether chickens showed aversive behavior in response to the CM extract. We prepared three doses of the CM extract solution, including 0.01% and 0.001% CM extract solutions that were the same doses as cell-based assays and an additional third dose of 0.1%. In the one-bowl drinking test, the intake volumes of 0.01% and 0.1% CM extract solutions were significantly lower than that of water (Fig. 2). However, chickens could not detect the bitterness in 0.001% CM extract (Fig. 2); this dose significantly increased the intracellular Ca^{2+} index in both cT2R1- and cT2R7-expressing cells. These results suggest that chickens sense the bitter taste of CM at the behavioral level.

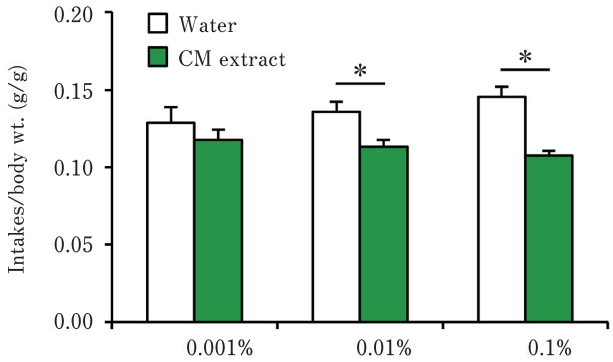

Fig. 2. **Behavioral analyses of the aversive responses to canola meal (CM) extract in chickens in a 10-min drinking test.** The intakes of 0.01% and 0.1% CM extract solutions were significantly lower than that of water. However, the chickens could not detect any bitterness in 0.001% CM extract solution compared to that in water. Data are the means±SE (n=8). * p<0.05 by paired t-test.

6-meth Inhibited the Cytosolic Ca^{2+} Responses to CM Extract

We previously reported that 6-meth is an antagonist for the functional bitter taste receptors (cT2R1 and cT2R7) in chickens (Dey *et al.*, 2017). In this research, we used the same compound to analyze whether 6-meth blocked the responses to CM extract via both cT2R1 and cT2R7. In Ca^{2+} imaging tests, although the RFU slightly increased for the first stimuli of the mixture (0.01% CM extract solution and 50 μM 6-meth), the RFU of the second stimuli (0.01% CM extract) increased more than that of the first stimuli (Fig. 3A and 3C). There were significant differences between the peak values of the first and second stimuli (Fig. 3B and 3D). However, in mock cells there were no responses to these solutions (Fig. 3E). These results suggest that 50 μM 6-meth can partially inhibit the responses to CM-extract-mediated cT2R1 and cT2R7.

6-meth Inhibited the Bitterness of CM Extract in Chickens

In the cell-based assays, 50 μM 6-meth inhibited the activities of 0.01% CM extract-solution-mediated cT2R1 and cT2R7. Thus, we examined whether 6-meth would inhibit the bitterness of the solution containing CM extract in vivo. In a one-bowl drinking test, addition of 50 μM 6-meth to 0.01% CM extract solution resulted in a significantly higher intake of the solution than that observed using 0.01% CM extract solution alone (Fig. 4). However, the addition of a lower concentration of 6-meth (25 μM) to 0.01% CM extract did not lead to any significant differences in intake during the 10-min drinking test, although the intake volume slightly increased by the addition of 25 μM 6-meth (p=0.064 by paired t-test) (Fig. 4). These results suggest that 6-meth can inhibit the bitterness of CM extract at the in vivo level.

Discussion

In this study, we found that CM contains agonists of both cT2R1 and cT2R7 by using both cell-based assays and be-

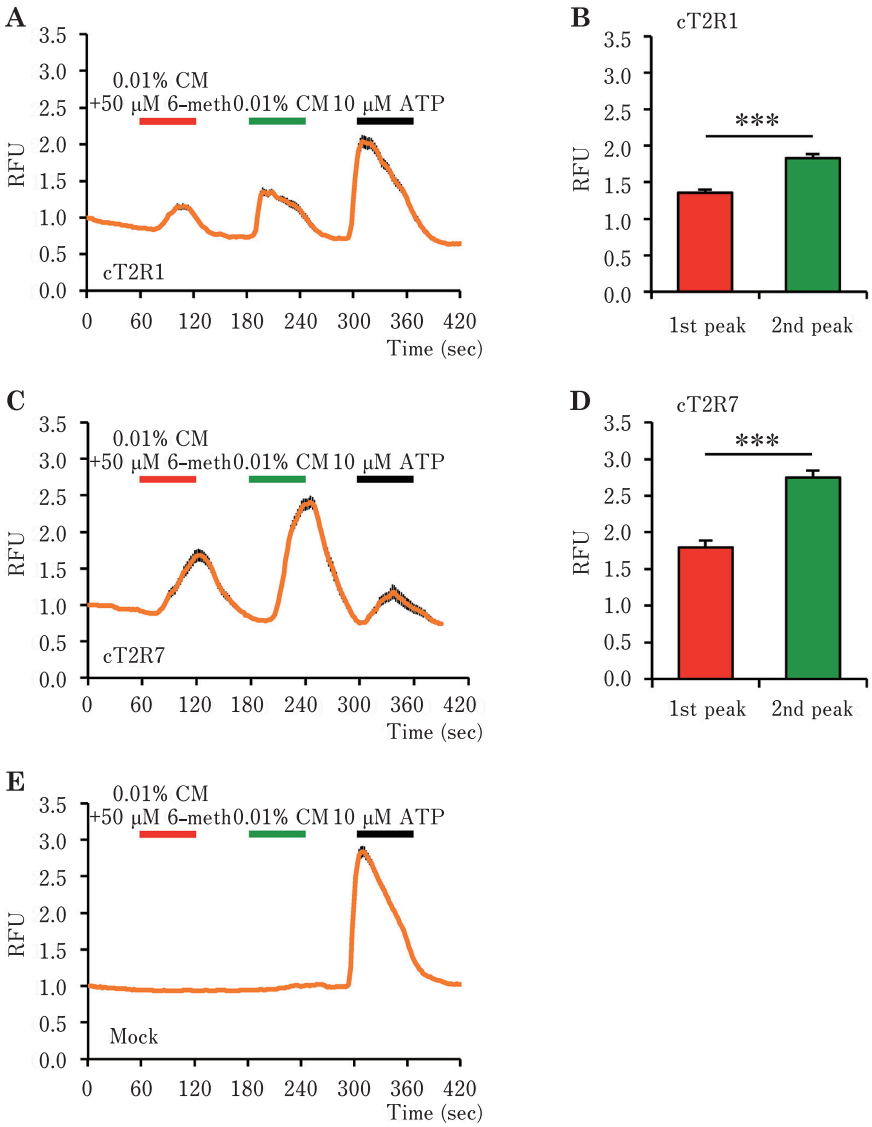

Fig. 3. **6-methoxyflavanone (6-meth) inhibited the activity of 0.01% canola meal (CM) extract, which contains the agonists of cT2R1 and cT2R7 in cT2R1- or cT2R7-expressing cells.** The representative data of the ratios of relative fluorescence units (RFUs), the index of cytosolic Ca^{2+} to the baseline value after stimulus with a mixture of 50 μM 6-meth and 0.01% CM extract solution, 0.01% CM extract solution only, and 10 μM ATP in (A) cT2R1/pDisplay- and G$\alpha_{16/gust44}$/pcDNA3.1(+)-expressing cells, (C) cT2R7/pDisplay- and G$\alpha_{16/gust44}$/pcDNA3.1(+)-expressing cells, and (E) mock (empty vector) cells. There were significant differences in the maximum RFU values between the mixture (1st peak) stimulus and the CM extract solution-only (2nd peak) stimulus in (B) cT2R1/pDisplay- and G$\alpha_{16/gust44}$/pcDNA3.1(+)-expressing cells and (D) cT2R7/pDisplay- and G$\alpha_{16/gust44}$/pcDNA3.1(+)-expressing cells. Data are the means±SE of 120−159 cells. *** $p<0.001$ by paired t-test.

havioral experiments. Further, we found that the bitterness of CM is inhibited by 6-meth, which was previously identified as an antagonist for the functional bitter taste receptors of chickens (Dey *et al*., 2017). We identified why the inclusion of high levels of CM in the diet reduces feed intake in chickens. Since 6-meth can improve the reduction of feed intake caused by the presence of CM, the present information will be useful to improve feedstuffs containing CM.

Chickens drank less CM extract solution than water in a 10 min drinking test. Since 10 min was insufficient time for the solution to be fully absorbed in the gastrointestinal tract, it is thought that chickens could detect the bitterness of the CM

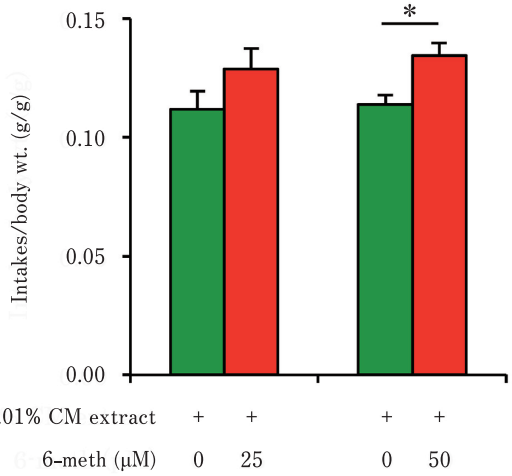

Fig. 4. **Behavioral analyses of the aversive responses to canola meal (CM) extract solution and the inhibition by 6-methoxyflavanone (6-meth) in chickens.** The intakes of 0.01% CM extract solution mixed with 50 μM 6-meth were significantly higher than that of 0.01% CM extract solution alone in the 10-min drinking test. Data are the means±SE (n=8). *p<0.05 by paired t-test.

extract in the oral tissues via both receptors cT2R1 and cT2R7. The dose-dependency of the CM extract in the behavioral drinking test showed that the chickens did not detect the bitterness of 0.001% CM extract solution, whereas they showed significant aversions to the higher concentrations of the CM extract. These results are reasonable because CM contains tannins, sinapine, and glucosinolates, which are naturally bitter. Unfortunately, many flavonoids have a negative effect on sensory perception because they are bitter (Drewnowski and Gomez-Carneros, 2000). The present results also showed the agonistic activities of CM for cT2R1 and cT2R7 and included the results of the behavioral test. However, the dose-dependency of CM was not observed in a cell-based assay (Fig. 1). The reason for this is possibly the desensitization of cT2R1 and cT2R7.

After confirming the responses to the bitterness of the CM extract both in vitro and in vivo, we examined whether the antagonist of the bitter taste receptors in chickens blocked the agonistic activity of CM. Previously, we had identified 6-meth as an antagonist for the functional bitter taste receptors in chickens (Dey *et al*., 2017). Thus, we assumed that 6-meth could also inhibit the bitterness responses of CM-mediated cT2R1 and cT2R7. We used 50 μM 6-meth to examine the antagonistic effect for cT2R1 and cT2R7 because this dose is effective for inhibiting other bitter compounds (Dey *et al*., 2017). In the present Ca^{2+} imaging tests, the antagonistic effects of 6-meth were partial. However, there were significant differences between first stimuli and second stimuli. In the behavioral tests, we confirmed the bitter inhibition effects of 6-meth was dose-dependent. As 50 μM 6-meth could inhibit the bitterness of CM at both in vitro and in vivo, the data of the cell-based assay were compatible with those of the behavioral test. These results were identical with those of our previous reports, which showed that cT2R1 activities were compatible with behavioral sensitivity to bitterness (Hirose *et al*., 2015), and the dose-dependencies of 6-meth in the behavioral tests almost matched those in the cell-based assay (Dey *et al*., 2017). Further, we confirmed that chickens did not show any aversion to 50 μM 6-meth itself compared to normal drinking water (Dey *et al*., 2017). So, 6-meth is not a substance that chickens can taste, and it can be used in the chicken feed industry to block the bitter compounds in nutritionally potential ingredients.

In the present study, we used both male and female chicks randomly; thus, we did not confirm the effects of sex on the sensitivity of 6-meth. Females are industrially important as egg layers; thus further studies are required to understand whether sex differences affect the taste sensitivities for CM and the effects of 6-meth in chicks. In this study, we prepared the CM extract by using DMSO; other compounds, which were not extracted by DMSO were not examined. The main bitter compounds of CM, such as tannin, sinapine, and glucosinolates are organic compounds, thus, it was considered that most of the main bitter compounds was extracted by DMSO. However, since CM might contain unknown water-soluble bitter compounds, it is important to examine the these compounds in future. Moreover, we did not confirm whether 6-meth can inhibit the bitterness of CM itself. Further studies are also needed to confirm that for developing poultry industry.

In summary, we have shown that that CM contains agonists for the functional bitter taste receptors in chickens and that 6-meth inhibited the bitterness of CM. These findings will be very useful in the development of new feedstuffs for chickens derived from CM. Further, as 6-meth is only one of the antagonists for the bitter taste receptors of chickens, it is necessary to explore other antagonists that may be economical and easier to use.

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Conflicts of Interest

The authors declare no conflict of interest.

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