JPSA
Japan Poultry Science Association

http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.0210132

≪Research Note≫

Identification of Ligands for Chicken Transient Receptor Potential Ankyrin 1 Channel and Chemosensory Perception of Herbal Compounds in Chickens

Fuminori Kawabata[1,2,*], Kana Murayama[1,*], Yuta Yoshida[1,3], Ruojun Liang[1], Shotaro Nishimura[1] and Shoji Tabata[1]

[1] Laboratory of Functional Anatomy, Faculty of Agriculture, Kyushu University, Fukuoka 819−0395, Japan
[2] Physiology of Domestic Animals, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki 036−8561, Japan
[3] Department of Food and Life Sciences, College of Agriculture, Ibaraki University, Ami 300−0393, Japan

The pungency induced by spices and herbs plays an important role in food choice and appetite, and it is suggested that adding spices and herbs to feed as natural alternatives to antibiotics has beneficial effects in poultry farming. However, our knowledge of the chemosensory perception of herbal compounds in chickens is limited. Transient receptor potential ankyrin 1 (TRPA1) is involved in the sensory perception of various herbal compounds. Here, we performed calcium imaging and electrophysiological analyses using cells transiently expressing chicken TRPA1 (cTRPA1) and identified two novel cTRPA1 ligands—eugenol and thymol. In a behavioral assay, chickens responded to cTRPA1 ligands, including eugenol, thymol, cinnamaldehyde, carvacrol, and allyl isothiocyanate. These results provide evidence that chickens have a functional TRPA1 channel and chemosensory perception of various herbal compounds.

Key words: chemosensory perception, chicken, herbs, TRPA1

J. Poult. Sci., 59: 286−290, 2022

Introduction

Aside from the senses of taste and smell, the pungency induced by spices and herbs plays a considerable role in food choice and appetite. Transient receptor potential (TRP) channels, such as TRP vanilloid 1 (TRPV1) and TRP ankyrin 1 (TRPA1), are involved in the chemosensory perception of pungent compounds (Rhyu *et al*., 2021). In mammals, TRPV1 is a pain receptor activated by capsaicin, a pungent substance present in hot chili pepper (Jordt and Julius, 2002), whereas TRPA1 is activated by a variety of natural spices and herbal compounds such as thymol, cinnamaldehyde (CA), carvacrol, and allyl isothiocyanate (AITC) (Rhyu *et al*., 2021). It is suggested that adding spices and herbs to poultry feeds enhances the performance of birds, improves feed utilization, helps digestion, and serves as natural alternatives to antibiotics (Vinus *et al*., 2018). Thus, identification of the ligands for chicken TRP channels will be an important step toward identifying novel feed additives in poultry farming. It has been reported that chicken TRPV1, unlike rat TRPV1, is hard for capsaicin to activate (Jordt and Julius, 2002), but CA, carvacrol, and AITC activate chicken TRPA1 (cTRPA1) (Saito *et al*., 2014). The aim of the present study was to identify novel cTRPA1 ligands using cell-based assays. Using a behavioral assay, we also investigated the effects of the identified ligands on the sensory perceptions of chickens.

Received: November 10, 2021, Accepted: December 8, 2021
Released Online Advance Publication: January 25, 2022
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)
* These authors contributed equally to this work

Materials and Methods

Chemicals

Eugenol, thymol, CA, carvacrol, and AITC were obtained from FUJIFILM Wako Pure Chemical Corp., Osaka, Japan. They were dissolved in dimethyl sulfoxide (DMSO) and stored at −20℃. Before the calcium imaging and patch clamp experiments, the DMSO stock solution (1 M) was 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 using NaOH. Moreover, before the brief-access test, the DMSO stock solution (1 M) was diluted using reverse osmosis water.

Cell Culture

Human embryonic kidney 293T (HEK293T) cells were

cultured in Dulbecco's modified Eagle's medium (high glucose; FUJIFILM Wako Pure Chemical Corp.) with 10% fetal bovine serum (GE Healthcare, Buckinghamshire, UK) and 1% penicillin–streptomycin (FUJIFILM Wako Pure Chemical Corp.) at 37℃ in 5% CO_2.

Calcium Imaging

Calcium imaging analyses were performed as described in our previous report (Liang *et al*., 2019). Briefly, HEK293T cells were transfected with either empty vector pcDNA3.1(+) for mock cells or cTRPA1/pcDNA3.1(+) using a lipofection method with ScreenFect A (FUJIFILM Wako Pure Chemical Corp.) on coverslips coated with poly-d-lysine (0.1 mg/ml; FUJIFILM Wako Pure Chemical Corp.). After transfection, the cells were incubated for 48 h at 37℃ in 5% CO_2. The cells were then incubated with Fluo4-AM solution, prepared according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan), for 30 min at 37℃ in 5% CO_2. The Fluo4-AM solution was then washed with bath solution, and the coverslips were mounted on a chamber connected to a gravity flow system to deliver various stimuli by running a bath solution containing various chemicals. Fluo4 fluorescence was measured using a laser scanning microscope (Nikon A1R; Nikon Solutions Co., Tokyo, Japan). The chemical stimuli tested in the calcium imaging analyses were 0.5 mM eugenol, 0.5 mM thymol, 0.5 mM CA, and 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). CA, a known cTRPA1 ligand (Saito *et al*., 2014), was used as a positive control.

Patch Clamp Recording

Whole-cell patch clamp recordings were performed as previously described (Liang *et al*., 2019). HEK293T cells were co-transfected with cTRPA1/pcDNA3.1(+) and EGFP/pCAGGS using ScreenFect A on coverslips coated with poly-d-lysine (0.1 mg/mL). The EGFP fluorescence was used to identify the successful cells in the transfection of cTRPA1. The cells were incubated for 24−48 h at 37℃ in 5% CO_2, and the coverslips were mounted in a chamber connected to a gravity flow system. The cells were voltage clamped at −60 mV using an EPC10 amplifier (HEKA Elektronik, Lambrecht, Germany). Patch pipettes, which have resistances between 3 and 5 MΩ, were filled with a pipette solution consisting of 140 mM KCl, 5 mM EGTA, and 10 mM HEPES at pH 7.4, adjusted with KOH. The chemical stimuli used in the patch clamp recording were 0.5 mM eugenol, 1.0 mM eugenol, 3.0 mM eugenol, 0.5 mM thymol, 1.0 mM thymol, and 0.5 mM CA.

Animals

The use of animals throughout the study was approved by the Committee for Laboratory Animal Care and Use at Kyushu University (approval no. A28-151-1) and followed 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 Japanese Government Notification on the Feeding and Safekeeping of Animals (Notification No. 6; March 27, 1980), and the ARRIVE guidelines. Fertilized eggs of the Rhode Island Red strain were obtained from the National Livestock Breeding Center's Okazaki station (Okazaki, Japan), and the chicks and their offspring were used for this experiment. The chicks were maintained in a box brooder with a heating system (Showa Furanki, Saitama, Japan) at a temperature of approximately 30℃ under a 12-h/12-h dark/light cycle. They were provided *ad libitum* access to commercial feed (Powerlayer 17Y, JA Kitakyushu Kumiai Shiryo, Fukuoka, Japan) and water.

Brief-access Test

Brief-access tests were performed as described previously with some modifications (Yoshida *et al*., 2021). Briefly, male and female chicks that were 0−2-week-old at the start of the experiment were used. The tests were performed over five consecutive days. Throughout the experiment, the chicks were supplied with commercial layer feed *ad libitum*, and water intake was restricted 23 h 40 min before the tests. Either the control solution or the test solution was presented to the chicks for 5 min, and then, to minimize the variance in the daily solution intake of each chick, normal tap water was presented for 15 min.

On days 1 and 2, the chicks were presented with the control solution for 5 min for a short-time intake training. On day 3, to get the chicks accustomed to the test solution and avoid neophobia, the chicks were presented with the test solution for 5 min. The test solutions used in the present study were as follows: 0.5−5.0 mM eugenol, 0.1−5.0 mM thymol, 0.1−5.0 mM CA, 0.5−5.0 mM carvacrol, and 0.05−5.0 mM AITC. On days 4 and 5, the chicks were randomly presented with the test or control solution. The control solution contained the same amount of DMSO as the test solution. The intake of each of the solutions was measured and compared to evaluate the chemosensory responses to the test stimuli. To evaluate the preferences for the test stimuli, the preference ratio for each solution was calculated as follows: test solution intake / (test solution intake + control solution intake), using the data on days 4 and 5, as previously reported (Yoshida *et al*., 2018a). For statistical analyses, each preference ratio was compared to each opposite ratio (control solution intake / [test solution intake + control solution intake]). A ratio >0.5 indicated preference, whereas a ratio <0.5 indicated aversion.

Statistical Analyses

Paired *t*-test was used for statistical analyses that were performed using Excel 2011 (Microsoft Corp., Redmond, WA, USA). Differences were considered significant at $P<0.05$.

Results

Eugenol and Thymol Activated cTRPA1

We performed calcium imaging analyses using cells transiently expressing cTRPA1 (cTRPA1 cells; Fig. 1A) or cells transfected with an empty vector (mock cells; Fig. 1B). Mock cells were not activated by 0.5 mM eugenol, 0.5 mM thymol, or 0.5 mM CA (Fig. 1B). By contrast, cTRPA1 cells were activated by 0.5 mM eugenol, 0.5 mM thymol, and 0.5 mM CA (Fig. 1A). Both mock and cTRPA1 cells were activated by 0.1% Triton X-100 (Figs. 1A, B), suggesting that the Fluo4 measuring system functioned normally in both cells. Electro-

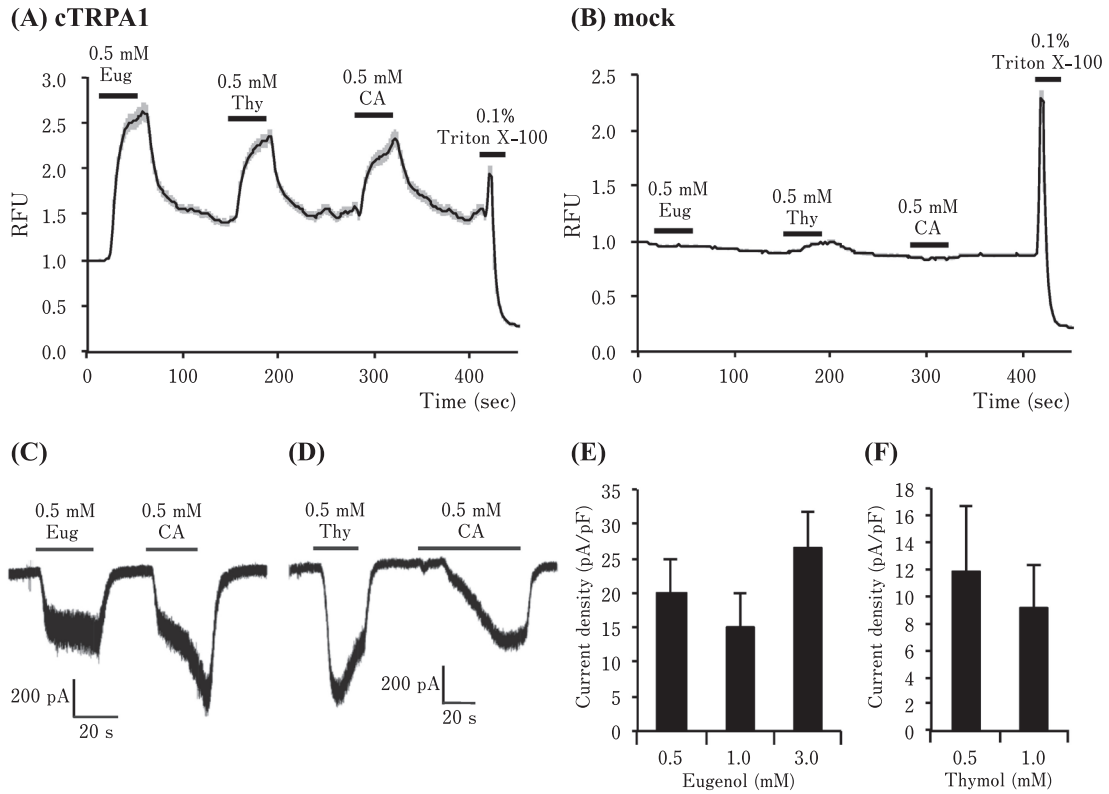

Fig. 1. **(A and B) Responses of HEK293T cells transiently expressing cTRPA1 (cTRPA1 cells) or cells transfected with empty vector (mock) to herbal compounds.** The ratios of relative fluorescence unit (RFU) that are the indices of cytosolic Ca^{2+} concentration after stimulation with 0.5 mM eugenol (Eug), 0.5 mM thymol (Thy), and 0.5 mM cinnamaldehyde (CA) in cTRPA1 cells (A) and mock cells (B) are presented. All cells responded to Triton X-100. Values are presented as the mean±SE. The data were obtained from 3 coverslips. Each had approximately 100 cells, and the averages of the data from these cells were treated as the data for one coverslip. (C−F) Electrophysiological responses of cTRPA1 cells to herbal compounds. The representative currents of cTRPA1 cells stimulated with 0.5 mM Eug and 0.5 mM CA (C) and 0.5 mM Thy and 0.5 mM CA (D) are shown. Current densities of cTRPA1 cells stimulated with 0.5 mM, 1.0 mM, and 3.0 mM Eug (E) and 0.5 mM and 1.0 mM Thy (F) are shown. Values are presented as the mean±SE. The data were obtained from 3 cells.

physiological analysis revealed that 0.5 mM eugenol, 0.5 mM thymol, and 0.5 mM CA activated cTRPA1 cells (Fig. 1C, D). At all concentrations examined in the whole-cell patch clamp tests, eugenol and thymol activated cTRPA1 (Figs. 1E, F).

Chickens Showed Chemosensory Responses to all the cTRPA1 Ligands Tested

Brief-access tests were performed to evaluate dose-dependent behavioral responses to five cTRPA1 ligands, namely, eugenol, thymol, CA, carvacrol, and AITC. We found that the 0.5 mM eugenol intake was not changed compared to that of the control solution ($P>0.05$), but the 1.0 mM and 5.0 mM eugenol intakes were decreased compared to that of the control solution (1.0 mM; $P<0.05$, 5.0 mM; $P<0.001$; Fig. 2A). Furthermore, the preference ratios for 1.0 mM and 5.0 mM eugenol were also decreased (1.0 mM; $P<0.05$, 5.0 mM; $P<0.001$; Fig. 2B). In addition, the 0.1 mM, 0.5 mM, and 1.0 mM thymol intakes were not changed compared to that of the control solution ($P>0.05$), but the 5.0 mM thymol intake was significantly decreased ($P<0.05$; Fig. 2C). Moreover, the preference ratio for 5.0 mM thymol was decreased ($P<0.01$; Fig. 2D). Furthermore, we also found that the 0.1 mM and 0.5 mM CA intakes were not changed, whereas the 1.0 mM and 5.0 mM CA intakes were decreased (1.0 mM; $P<0.05$, 5.0 mM; $P<0.001$; Fig. 2E). The preference ratio for 5.0 mM CA was also significantly decreased ($P<0.001$; Fig. 2F). Both the 1.0 mM and 5.0 mM carvacrol intakes were significantly decreased (1.0 mM; $P<0.05$, 5.0 mM; $P<0.001$; Fig. 2G), and the preference ratios for 1.0 mM and 5.0 mM carvacrol were also decreased (1.0 mM; $P<0.05$, 5.0 mM; $P<0.001$; Fig. 2H). We also found that the 0.1 mM AITC intake was not changed ($P>0.05$), but the 0.05 mM, 0.5 mM, 1.0 mM, and 5.0 mM AITC intakes were significantly decreased compared to that of the control solution (0.05 mM; $P<0.05$, 0.5 mM; $P<0.01$, 1.0 mM; $P<0.001$, 5.0 mM; $P<0.001$; Fig. 2I). The preference ratios for 0.5 mM, 1.0 mM, and 5.0 mM AITC were significantly decreased (0.5 mM; $P<0.01$, 1.0 mM; $P<0.001$, 5.0 mM; $P<0.001$; Fig. 2J).

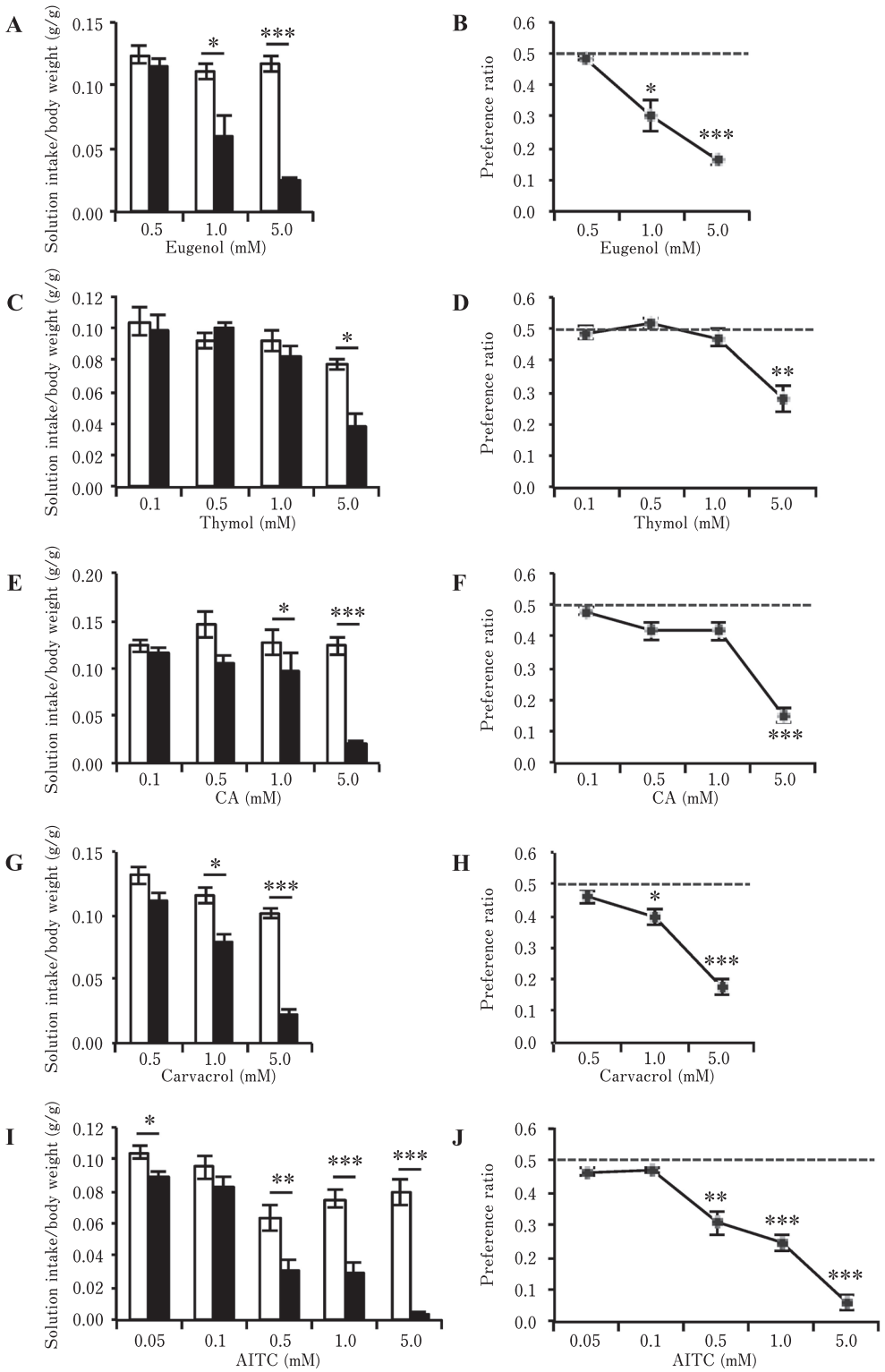

Fig. 2. **Behavioral responses to various herbal compounds in chickens.** (A, C, E, G, and I) Control solution intake/BW (white bar) and flavored test solution intake/BW (black bar) were compared using 0.5 mM, 1.0 mM, and 5.0 mM eugenol (A); 0.1 mM, 0.5 mM, 1.0 mM, and 5.0 mM thymol (C); 0.1 mM, 0.5 mM, 1.0 mM, and 5.0 mM cinnamaldehyde (CA) (E); 0.5 mM, 1.0 mM, and 5.0 mM carvacrol (G); and 0.05 mM, 0.1 mM, 0.5 mM, 1.0 mM, and 5.0 mM allyl isothiocyanate (AITC) (I). (B, D, F, H, and J) Preference ratios for the corresponding concentrations of eugenol (B), thymol (D), CA (F), carvacrol (H), and AITC (J) are shown. Values are presented as the mean±SE (n=6–8). * P<0.05, ** P<0.01, and *** P<0.001 using paired t-test.

Discussion

The results of the calcium imaging assay and electrophysiological analysis revealed that the natural herbal compounds eugenol and thymol activated cTRPA1 similarly to the known cTRPA1 ligand CA (Saito *et al*., 2014). Because these stimuli did not affect the mock cells in the calcium imaging, it was considered that these herbal compounds specifically activated the cTRPA1 channel without affecting any endogenous receptors in HEK293T cells. In addition, the brief-access test demonstrated that the cTRPA1 ligands eugenol, thymol, CA, carvacrol, and AITC (Saito *et al*., 2014) induced behavioral responses in chickens. Thus, the present study identified two novel cTRPA1 ligands and demonstrated that chickens have chemosensory perception of natural herbal compounds that activate cTRPA1. Furthermore, it should be noted that AITC activates mouse TRPV1, but not chicken TRPV1 (Kawabata *et al*., 2017), suggesting that the behavioral responses to AITC observed in the present study can be mediated by cTRPA1. Since the brief-access test was conducted to minimize post-ingestive effects (Yoshida *et al*., 2018b), it was hypothesized that chickens detected the herbal compounds by olfactory and/or oral trigeminal chemoreception. In mammals, TRPA1 is expressed in sensory neurons, including the olfactory receptor neurons and trigeminal nerve fibers (Nakashimo *et al*., 2010; Rhyu *et al*., 2021). Thus, future immunohistochemical analyses on the localization of TRPA1 in chicken sensory neurons will contribute to our understanding of the chemosensory perception of spices and herbal compounds in chickens.

We observed that the chickens significantly rejected the high concentrations of the cTRPA1 ligands in the brief-access test, although the concentrations that repelled them in the behavioral tests were slightly above the concentrations that activated cTRPA1 in the patch clamp tests. Because trigeminal nerve fibers expressing cTRPA1 may be covered with a multilayered epithelium, there may be gaps in effective concentrations between channel functions and behaviors. In a previous study, cTRPA1 was activated by the bird repellent methyl anthranilate (Saito *et al*., 2014). Thus, it was assumed that eugenol and thymol identified in the present study could be potential non-lethal bird repellents. However, a study on broilers suggested that supplementation with an essential oil mix from oregano and clove, containing eugenol and thymol, improved the feed conversion ratio even more than in the group supplemented with only an antibiotic (Ertas *et al*., 2005). This suggested the possibility that an adequate concentration of herbal compounds could improve chicken preferences and performance. Therefore, further studies are needed to investigate the preferences for these herbal compounds when added to feed, as well as their effects on appetite, for the future use of cTRPA1 ligands in poultry farming.

Acknowledgments

We thank Dr. Makoto Tominaga and Dr. Shigeru Saito (National Institute for Physiological Sciences, National Institutes of Natural Sciences) for kindly providing the cTRPA1 construct. This study was supported by a grant to Fuminori Kawabata from JSPS KAKENHI (#26850207).

Author Contributions

Fuminori Kawabata, Kana Murayama, Yuta Yoshida, Ruojun Liang, Shotaro Nishimura, and Shoji Tabata designed the experiments. Kana Murayama and Ruojun Liang conducted the experiments. Yuta Yoshida, Fuminori Kawabata, and Kana Murayama drafted the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

Ertas ON, Guler T, Çiftçi M, DalkIllç B and Simsek UG. The effect of an essential oil mix derived from oregano, clove and anise on broiler performance. International Journal of Poultry Science, 4: 879−884. 2005.

Jordt SE and Julius D. Molecular basis for species-specific sensitivity to “hot” chili peppers. Cell, 108: 421−430. 2002.

Kawabata F, Kawabata Y, Liang R, Nishimura S and Tabata S. Comparative analysis of allyl isothiocyanate (AITC)-induced carbohydrate oxidation changes via TRPV1 between mice and chickens. Biomedical Research (Tokyo), 38: 149−155. 2017.

Liang R, Kawabata Y, Kawabata F, Nishimura S and Tabata S. Differences in the acidic sensitivity of transient receptor potential vanilloid 1 (TRPV1) between chickens and mice. Biochemical and Biophysical Research Communications, 515: 386−393. 2019.

Nakashimo Y, Takumida M, Fukuiri T, Anniko M and Hirakawa K. Expression of transient receptor potential channel vanilloid (TRPV) 1–4, melastin (TRPM) 5 and 8, and ankyrin (TRPA1) in the normal and methimazole-treated mouse olfactory epithelium. Acta Oto-Laryngologica, 130: 1278−1286. 2010.

Rhyu MR, Kim Y and Lyall V. Interactions between chemesthesis and taste: Role of TRPA1 and TRPV1. International Journal of Molecular Sciences, 22: 3360. 2021.

Saito S, Banzawa N, Fukuta N, Saito CT, Takahashi K, Imagawa T, Ohta T and Tominaga M. Heat and noxious chemical sensor, chicken TRPA1, as a target of bird repellents and identification of its structural determinants by multispecies functional comparison. Molecular Biology and Evolution, 31: 708−722. 2014.

Vinus RD, Sheoran N, Maan N and Tewatia B. Potential benefits of herbal supplements in poultry feed: A review. The Pharma Innovation Journal, 7: 651−656. 2018.

Yoshida Y, Kawabata F, Kawabata Y, Nishimura S and Tabata S. Expression levels of taste-related genes in palate and tongue tip, and involvement of transient receptor potential subfamily M member 5 (TRPM5) in taste sense in chickens. Animal Science Journal, 89: 441−447. 2018a.

Yoshida Y, Kawabata F, Kawabata Y, Nishimura S and Tabata S. Short-term perception of and conditioned taste aversion to umami taste, and oral expression patterns of umami taste receptors in chickens. Physiology & Behavior, 191: 29−36. 2018b.

Yoshida Y, Kawabata F, Tabata S, Aggrey SE, Rekaya R and Liu HX. Evolvement of taste sensitivity and taste buds in chickens during selective breeding. Poultry Science, 100: 101113. 2021.