MOLECULAR CHARACTERIZATION AND EXPRESSION OF UMAMI RECEPTORS T1R1/T1R3 IN BROILER CHICKENS

BY

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ABSTRACT

Taste receptors detect nutrient substances and toxic compounds and are essential in animals for maintaining body health and energy balance. Chickens can recognize four out of five basic tastes including bitter, sour, salty, and umami tastes but not sweet. Umami is a pleasant taste that is responsible for recognizing protein and has been found in the chicken genome. Recent studies have found that these receptors in the gut function as chemosensors for detecting chemical signals of luminal nutrients. However, the location and pattern of development in the gut umami taste receptor is unknown in chickens. This study aims to investigate the localization of umami taste receptors and their expression in the gut during embryonic and post-hatch development. Our results showed that umami taste receptors are widely expressed in the different tissues and in the intestine during late embryonic and post-hatch development in broiler chickens. The jejunum had the highest ($P < 0.05$) expression level of umami receptors in the intestine in 35d broiler chickens. Furthermore, jejunal T1R1/T1R3 mRNA abundance increased with age during late embryonic development in chickens. The expression of T1R1 in the duodenum and jejunum changed quadratically ($P < 0.05$), while the linear and quadratic patterns of changes ($P < 0.05$) were found in the colon during post-hatch development. These results demonstrated that chicken umami receptors T1R1/T1R3 are expressed in the intestine in the late embryonic stage and post-hatching stage and their level of expression may have been affected by the ages of embryonic and post-hatch development. In order to gain further insight into the potential ligands of chicken umami receptors, molecular docking was followed. Ligand docking results showed that chicken T1R1/T1R3 may be broadly tuned by L-amino acids, peptides, and nucleotides. Further *in vitro* and *in vivo* studies are needed to validate docking results and to investigate cell signaling pathways activated by potential umami substances. These findings

may have profound implications for novel therapeutic and new feed additives innovation based on the gut expression pattern of chickens during development.

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DEDICATION

This thesis is dedicated to my parents, Xianzhong Dong and Yali Cheng, for always loving and supporting me.

FOREWORD

Part of this thesis was presented as a poster presentation at the 2020 Animal Nutrition Conference of Canada Virtual Conference Meeting and an oral presentation at the 2020 Poultry Science Association Annual Meeting. The abstracts and manuscripts prepared during my MSc. program are listed as follows:

1. Dong, B.*, Kim, E., Kiarie, E. G., Yang, C. Expression of umami receptors (T1R1/T1R3) in the intestine during the embryonic and post-hatch development in broiler chickens (Poster presentation). 2020 Animal Nutrition Conference of Canada, Webinar Series, May 26-28, 2020.

2. Dong, B.*, Kim, E., Kiarie, E. G., Yang, C. Expression of umami receptors (T1R1/T1R3) in the intestine during the embryonic and post-hatch development in broiler chickens (Oral Presentation). 2020 Poultry Science Association Annual Meeting, Online, July 20-22, 2020.

3. Dong, B.*, Zhao, X. *, Kim, E., Ni, L., Liu, Y., Nguyen, T., Li, T., Tang, M., Kiarie, E. G., Gong, J., Chen, G., Yang, C. (2020). Expression of umami receptors (T1R1/T1R3) in the intestine during the embryonic and post-hatch development in broiler chickens. Journal of Agricultural and Food Chemistry. Manuscript under preparation.

4. Kim, E., Akhtar, N., Li, J., Hui, Q.*, Dong, B.*, Yang, C., Kiarie, E. G. (2020). *In ovo* feeding of epidermal growth factor (EGF): embryonic expression of intestinal EGF receptor and post-hatch growth performance and intestinal development in broiler chickens. Poultry Science, 99(11): 5736-5743.

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LIST OF ABBREVIATIONS

AA	Amino acid
ASIC	Acid-sensing ion channel
β-actin	Beta-actin
Blast	Basic local alignment search tool
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CaSR	Calcium-sensing receptor
CAT-1	Cationic amino acid transporter 1
CBF	Ciliary beat frequency
CCK	Cholecystokinin
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CRD	Cysteine-rich domain
DAG	Diacylglycerol
EAAT3	Excitatory amino acid transporter 3
ECD	Extracellular transmembrane domain
EEC	Enteroendocrine cell
ENaC	Amiloride sensitive epithelial sodium channel

ER	Endoplasmic reticulum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine 5’-diphosphate
GIT	Gastrointestinal tract
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon-like peptide-2
GLUT5	Glucose transporter 5
GTP	Guanosine 5’-triphosphate
GPCR	G protein-coupled receptor
HCL	Hydrochloric acid
HEK	Human embryo kidney
ICD	Intracellular transmembrane domain
IMP	Inosine 5’-monophosphate
IMINO	Transporter of proline and hydroxyproline in intestine/kidney
IP	Intraperitoneal administration

IP_3	Inositol triphosphate
KO	Knockout
LBD	Ligand binding domain
mGluRs	Metabotropic glutamate receptors
MTORC1	Mammalian target of rapamycin complex 1
MSG	L-monosodium glutamate
Na^+	Sodium ion
NaCl	Sodium chloride
NK	Natural killer cells
Otop1	Otopetrin 1
PCR	Polymerase chain reaction
PepT1	Peptide transporter 1
PLC β2	Phospholipase C β2
PTC	Phenylthiocarbamide
PKD	Polycystic kidney disease protein
PYY	Peptide tyrosine tyrosine
rmsd	Root-mean-square deviation
RNA	Ribonucleic acid

SGLT1	Sodium-dependent glucose co-transporter 1
STC-1	Intestinal secretin tumor cell line
TRCs	Taste receptor cells
TRPM5	Transient receptor potential cation channel subfamily M member 5
TSC	Taste sensory cells
T1R1	Taste receptor type 1 member 1
T1R2	Taste receptor type 1 member 2
T1R3	Taste receptor type 1 member 3
T2R	Bitter taste receptor
TM	Transmembrane
TMD	Transmembrane domain
Val	Valine
VFT	Venus fly trap
3D	Three-dimensional

CHAPTER 1 GENERAL INTRODUCTION

The sense of taste in animals is essential for distinguishing nutrient compounds from harmful substances, and ultimately regulating food intake and health conditions. Animals sense nutritional substances by stimulating taste receptor cells (TRCs), taste buds and taste nerves to initiate a combined effect of the chemosensory signals and physiological systems. The nutritional importance of the chemosensing system is a biological process that is related to molecular mechanism changes in genomic, metabolism, animal behaviour and physiological function (Niknafs and Roura, 2018; Roura et al., 2016). In mammals and avian species, taste perception has direct implications in energy balance and the discrimination of nutrients from toxic substances. Mammals have a fully functional olfactory sensing system to recognize five basic tastes, including sweet, bitter, umami, salty, and sour. Previous studies reported that avian species such as chickens have a well-developed sense of taste and can distinguish at least six tastes: bitter, umami, salty, sour, fatty, and kokumi (Cheled-Shoval et al., 2015; Dey et al., 2018; Niknafs and Roura, 2018; Yoshida et al., 2015). However, chickens lack sweet taste receptors, and the taste sensing of fatty acid is still peculiarly controversial (Cheled-Shoval et al., 2015; Roura et al., 2012). Kokumi, an enhancer of basic taste, has been recently discovered, indicating that chickens might have a specific taste receptor to sense this newfound flavour (Maruyama et al., 2012).

Sweet (T1R2/T1R3), bitter (T2R family), umami (T1R1/T1R3), and kokumi (calcium-sensing receptor) taste receptors are chemosensory receptors pertaining to class C G-coupled protein receptors (GPCRs), whereas sour and salty taste are controlled by ion channels such as the epithelial sodium channel (ENaC), the acid-sensing ion channel (ASIC), and the polycystic kidney disease protein (PKD) (Vi and Obrist, 2018; Yoshida et al., 2005). Taste receptors were initially found in the apical surface of the taste cells in taste buds that activate taste transduction in responding to

many chemical stimuli to guide animals for food selection. However, recent studies reveal that the expression and the function of taste receptors are not only present in the lingual tissues but also in non-lingual tissues from the respiratory tract, pancreas, and gut, suggesting that taste receptors may have profound implications in poultry nutrition. This review aims to corroborate the impact of the physiological function of the umami, bitter, sour, salty and kokumi taste system in chickens, as well as determine how the taste receptors sense luminal molecules and activate the secretion of hormone neurotransmitters in the gut.

CHAPTER 2 LITERATURE REVIEW

2.1 TASTE PERCEPTION IN POULTRY

2.1.1 Physiology of the taste system in poultry

2.1.1.1 Sweet

Sweet is a pleasant taste elicited by sugars and artificial sweeteners. Unlike humans and mammals, chickens are sweet-insensitive. Recent discoveries of the sweet taste receptor gene T1R2 have shown that this gene is conservative in most vertebrates except cats and birds who lack a functional T1R2 gene (Li et al., 2006). Chickens lack the T1R2 gene from their genome which is the receptor structure responsible for the assembly of T1R3 receptors which detect a sweet taste. However, chickens do show preference to sucrose, fructose, and xylose in varying degrees (Cheled-Shoval et al., 2015; Gentle, 1971). Halpern (1962) indicated that chickens refuse xylose solution but choose sucrose under some conditions (Halpern, 1962). Using the same assay, the threshold of L-monosodium glutamate (MSG) (the umami substance) and sucrose for broiler chickens was reported to be 300mM and 1M respectively, suggesting that chickens are less sensitive to sucrose than to MSG (Cheled-Shoval et al., 2017). A short-term trial was conducted to eliminate the post-ingestive effect, and it indicated that chickens fed a low-calorie diet showed a stronger preference for a 10% sucrose solution compared to chickens fed a regular diet (Kare and Maller, 1967). On the other hand, electrophysiological techniques indicated that chicken taste buds do not have impulse response when treated with sucrose or saccharine (Duncan, 1960; Halpern, 1962). Post-hatch chicks showed no discriminative preference to different sweet stimuli in the behaviour test as well (Ganchrow et al., 1990). Taken together, chickens do not significantly respond to sweet stimuli unless a high concentration of sugary substances is provided, likely due to lacking the T1R2 receptor. The reason why chickens lost the T1R2 gene would be an interesting topic for

investigation. Some preferences of sweet stimuli in chickens are probably because of nutrient and energy requirements for body maintenance or metabolism.

2.1.1.2 Bitter

Bitter is a taste that could be used as an indicator of toxic compounds that would induce aversive responses in animals. It is well known that chickens sense bitter compounds by T2R families including T2R1, T2R2, and T2R7 belonging to Class A GPCRs (Di Pizio and Niv, 2014). Although chickens have fewer bitter receptors than other vertebrates, this does not reduce the functionality for bitter sensation since bitter taste receptors are finely tuned (Niknafs and Roura, 2018). Chickens have a high tolerance for the bitter taste which is different than mammals. For example, sucrose octaacetate tastes bitter to humans, but it does not taste bitter to chickens (Kare, 1970). On the contrary, quinine chloride, a bitter substance for humans, produces an aversive response while activating all three bitter taste receptors in chickens (Cheled-Shoval et al., 2014; Kare, 1970). The level of exposure to bitter elements varies with age and concentration. Dey et al. (2018), using a behavioural drinking test, demonstrated that the bitter sensation changes in an age-dependent manner, that is, an 8-9-week old chicken was more sensitive to the bitter taste than were newly hatched chicks (Dey et al., 2018). The aversive responses to bitter stimuli from chickens are dose-dependent, which has also been reported in mammals (Liu et al., 2018).

2.1.1.3 Salty

The salty taste can stimulate positive or negative ingestion responses based on the level of concentration. A high level of NaCl may elicit an aversive response, but a low concentration can improve appetite and growth (Hurley et al., 2014). Chickens are more tolerant of a salty taste

compared to humans and other mammals. Salt substances such as sodium chloride ($NaCl_2$) are recognized by the epithelial sodium channel (ENaC) in type I taste cells present in the taste cell membrane of taste buds (Yarmolinsky et al., 2009). In mammals and rodents, the salty taste receptors are proposed to be transmembrane channels for hydrogen ions (Roura et al., 2012). Besides, the orthologous genes of both ENaC and hydrogen ion channels were found in chicken genome databases, suggesting salty taste receptors may also be conserved in poultry (Roura et al., 2012). The salty taste sensation is correlated with ages in poultry (Narukawa et al., 2017). Behaviour assays showed that older mice liked salty less than young mice, but other researchers reported that the salty and sweet taste sensations increased with age (Narukawa et al., 2017). Similarly, salty and sour tastes are also growth-associated in chickens, since newborn chicks are more sensitive to the salty taste than adult birds (Dey et al. 2018).

2.1.1.4 Sour

Acid-sensing is vital in the animal taste system and responsible for distinguishing raw food from rotten food and regulating the homeostasis of body fluids (Huang et al., 2006). There are a few acid-sensitive or proton selective ion channels that have been confirmed to sense sour tastants (Richter et al., 2004) such as PKD2L1, ASIC-2 and Otop1 (Teng et al., 2019). The polycystic kidney disease ion channel (PKD) and 2L1/PKD1L3 channels in type III taste cells are potential taste receptors for triggering the sour taste (Chandrashekar et al., 2006; Yarmolinsky et al., 2009). Huang et al. (2006) reported that PKD2L1 expression in TRCs has a unique function to mediate sour taste since the ablation of PKD2L1-expressing cells eliminates the taste response to sour (Huang et al., 2006). However, the knock-out of either the PKD2L1 or PKD1L3 gene in mouse TRCs did not affect the function of sour taste (Ye, 2016). Thus, it is yet unclear if PKD2L1 and PKD1L3 are the

only receptors to mediate acid perception. The acid-sensing ion channel-2 (ASIC-2) has been identified as the sour taste receptor candidate in rat taste cells, but not in mice. Richter et al. (2004) found that the knockout of ASIC-2 in mice did not disrupt the normal physiological function to acid taste stimuli (Richter et al., 2004). Another sour receptor candidate, the proton selective ion channel Otop1, is located in type III TRCs (Teng et al., 2019). In particular, Otop1-KO mice showed significant decreases in cellular and gustatory responses to sour stimuli, suggesting that Otop1 functions as an acid-sensing receptor (Teng et al., 2019). Chickens are more tolerant of sourness as they only respond to a high concentration of sour stimulation (Liu et al., 2018). For example, chickens can consume acidic solutions at a low pH of 1.5, but they show a preference for food with a higher pH (Kare, 1970). A recent study by Zocchi et al. (2017) found that mammals sense water by acid-sensing taste receptor cells which were previously reported as the sour taste receptor (Zocchi et al., 2017). However, the functions of the ion channels in chickens to sense the sour taste are unknown and require further investigation. Further research is required to clarify the functions of the ion channels for sour taste in chickens.

2.1.1.5 Kokumi

Kokumi does not belong to the five basic tastes nor have taste itself, but it provides continuity, mouthfulness, and thick flavour (Hartley et al., 2019). Kokumi was recently discovered as a pleasant taste recognized by the calcium-sensing receptor (CaSR) and triggered transduction pathways by calcium and amino acids (Wellendorph et al., 2010). A recent study indicated that CaSR activity was activated by glutathione and γ-Glu-Val-Gly (kokumi peptide), which can improve the intensity of kokumi flavour. On the other hand, the kokumi intensity was significantly decreased by a specific CaSR inhibitor, NPS-2143 (Kuroda and Miyamura, 2015). Furthermore, an

augmentation of sweetness, saltiness, and umami was observed when 3.3% sucrose, 0.9% NaCl and 0.5% MSG solution with γ-Glu-Val-Gly was provided (Kuroda and Miyamura, 2015). Taken together, kokumi substances might function as an enhancer for umami, sweet and salty taste. From a commercial perspective, there has been an increased interest in the dietary calcium appetite of poultry since sufficient calcium is essential for egg production and bone stores (Tordoff, 2001). Tordoff demonstrated that calcium-deprived chickens showed an enhancement of appetite and palatability of calcium, suggesting calcium signaling transduction in chicken taste cells (Tordoff, 2001). However, whether the CaSR is a taste receptor for mediating calcium appetite and palatability in chickens still needs to be discovered.

2.1.1.6 Umami

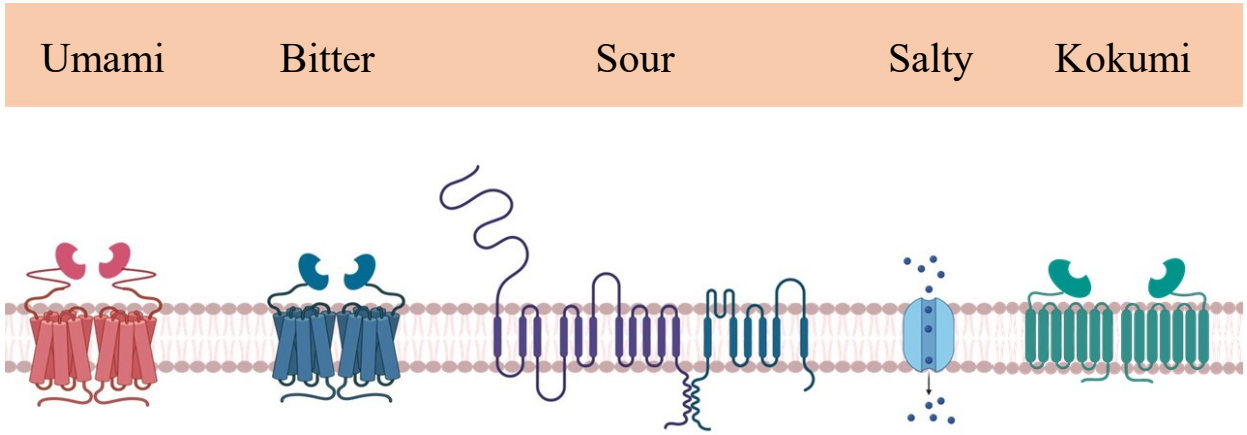
Umami is a savoury taste and plays an important role in detecting a wide range of L-amino acids in poultry and most mammalian species. In humans, the umami sensation is specifically evoked by monosodium glutamate and aspartate (Chandrashekar et al., 2006). Umami taste receptors T1R1 and T1R3 belong to class C GPCRs (Yoshida et al., 2015). Sweet and umami taste receptors share the T1R3 subunit, but they each combine with different T1R families which makes them into two different molecules (Torrallardona and Roura, 2009). Our understanding of umami receptors residing in the tongue and extraoral tissues focusing on humans and mice has been greatly advanced in the past decade, yet the role of T1R1/T1R3 in the chicken gut still needs to be investigated.

A recent study unveiled that the amino acid sensed by T1R1/T1R3 triggers tumoricidal activity and natural killer (NK) cell-mediated cytotoxicity on tumour cells through umami taste receptors (Liu et al., 2018). Daly et al. (2013) investigated the functional properties of the umami taste receptor using the STC-1 mouse enteroendocrine cell line. These authors reported that

T1R1/T1R3 heterodimer act as a luminal sensor to detect L-amino acid (e.g. Phe-, Leu-, and Glu-) and induce cholecystokinin (CCK) (Daly et al., 2013). CCK, a peptide hormone secreted by the gastrointestinal tract, is responsible for lipid and protein digestion which is expressed mainly in the chickens' ileum (Reid and Dunn, 2018). These findings elucidate that the umami taste receptors may have application functions to regulate food intake and gut health by luminal amino acids.

2.1.2 Taste receptors in poultry

It has been widely understood that chickens have fewer genes for taste receptors compared to mammals. For example, chickens have the umami taste receptors T1R1/T1R3 but lack the sweet taste receptor T1R2; also, the bitter taste receptors form only three subfamilies in chickens. Sweet, umami, bitter, and kokumi tastes are mediated by GPCRs in TRCs, while salty and sour taste machinery is thought to be mediated by ENaC and hydrogen ion channels respectively (**Fig. 2.1**) (Roura et al., 2012).



T1R1+T1R3	T2R1/T2R2/T2R7	PKD2L1/PKD1L3 (ASIC-2/Otop1)	ENaC	CaSR

Figure 2.1. A summary of taste sensations and receptors in poultry

It is well known that taste receptors function as part of the chemosensing system, where they sense nutritional molecules or ligands to trigger downstream signaling pathways on cell membranes and initiate afferent neuron signals to the brain, thus resulting in taste sensation. The taste receptors located on the cell membrane are responsible for certain taste stimuli; however, other chemicals can also penetrate cell membranes such as sodium, protons, bitter compounds, and sugars. These substances may interact with secondary messengers in intracellular membranes in order to activate TRCs. Therefore, a clear definition of taste receptors for those ligands is needed.

Recently, a few proteins were believed to function as a taste receptor, but not all were accepted as such without objection. To investigate the identity of candidate taste receptors, four aspects must be considered: 1) the protein function and its molecular identity of taste receptor should be defined; 2) the expression of taste receptors in TRCs must be investigated; 3) the corresponding ligands should be identified; and 4) whether alterations on the taste receptors cause the function to change in taste perception should be determined (Bachmanov and Beauchamp, 2007).

Whether the functions of the extraoral taste receptors work differently than those in oral tissues is not known. Emerging studies showed that taste receptors play an important role in the airway innate immune system as well as in the host-microbiota interaction, which will be further discussed in the next section.

2.1.3 Tissue distribution of taste receptors in poultry

2.1.3.1 Oral tissues

Taste buds in the oral cavity are the main sensory organs for detecting different taste perceptions and conducting gustatory stimuli to the brain through neural signals. The different types of taste receptors and ion channels were first discovered in taste receptor cells in the tongue epithelium that constitute the taste buds. The shape of taste buds varies among species. Mammals have an onion-shaped taste bud structure, which is different from chickens whose taste buds are ovoid shaped (Liu et al., 2018). In chicken oral tissues, the distribution of taste receptor cells in the epithelial tissue of the oral cavity was discovered in three regions: in the palate epithelium (~69%), in the base of the oral cavity (~29%), and in the posterior side of the tongue (~2%) (Ganchrow and Ganchrow, 1985). The number of taste buds is species dependent. Despite the relatively low taste bud numbers in broiler chickens (a total of 507 in the palate and 260 in the base of the oral cavity) compared to other species, as shown in **Table 2.1**, the volume of taste buds ratio is high in the oral cavity in chickens (Ganchrow and Ganchrow, 1985; Rajapaksha et al., 2016). Furthermore, the number of taste buds in chickens varie among strain/breed. For example, broiler-type chickens have almost twice as many taste buds in the oral cavity as layer-type chickens, and more taste buds were observed in the broiler female line than in males (Rajapaksha et al., 2016). Therefore, chickens have developed functioning taste receptors in their oral tissue, although chickens have small oral cavities compared to those of other species.

Table 2.1. Summary of taste bud numbers in oral cavity among vertebrate species

Species	Taste buds	References
Broiler chicken	767	(Rajapaksha et al., 2016)
Pigeon	59	(Duncan, 1960)
European bullfinch	46	(Wenzel, 1973)
Japanese quail	62	(Wenzel, 1973)
Lizard	550	(Schwenk, 1985)
Hamster	723	(Miller and Smith, 1984)
Domestic cat (adult)	2755	(Robinson and Winkles, 1990)
Dog	1706	(Leibetseder, 1980)
Human	2,000~5,000	(Carlson, 2019)
Pig	19,904	(Chamorro et al., 1993)
Cow	25,000	(Kare, 1970)
Catfish	100,000	(Hyman, 1943)

In *in vitro* studies, α-Gustducin and Vimentin, molecular markers used to characterize chicken taste buds from embryonic day (E) 17 to post-hatching d5, were observed in all the taste buds. Furthermore, α-Gustducin immunosignals were present in all taste buds in embryonic and post-hatch stages, but Vimentin became more apparent in chicken taste buds after hatch (Venkatesan et al., 2016). Each taste bud is a separate sensory organ consisting of 50-100 spindle-shaped taste cells (**Fig. 2.2**), and each taste cell has different functions for tastants (Roper, 2016). Molecular markers assay showed there are at least three types of taste sensory cells (TSC) in each taste bud: Type I, Type II (sweet, umami, bitter and kokumi taste cells) and Type III (sour, salty,

and kokumi taste cells) (Chandrashekar et al., 2006; Maruyama et al., 2012). Sweet, bitter, umami, and kokumi taste in type II taste sensory cells are stimulated by taste receptors, T1R, T2R, and CaSR (Roura et al., 2012). The localization of kokumi taste receptors in taste cells is still controversial. One study reported that the agonists of CaSR only respond in type III cells (Bystrova et al., 2010), which is in contrast to another paper which showed that all agonists of CaSR could be activated by both type II and type III cells (Maruyama et al., 2012).

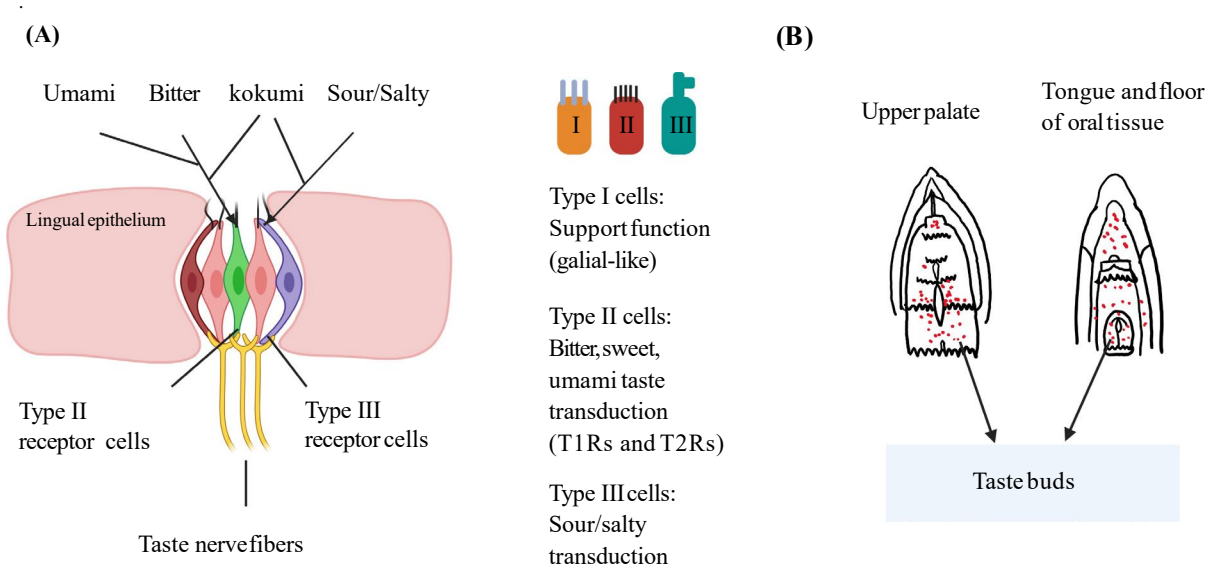


Figure 2.2. The distribution and structure of taste buds (767 in total) in the upper palate and oral cavity of broiler chickens (Rajapaksha et al., 2016). Red dot=taste bud. (A) shows the taste bud structure, and (B) shows the localization of taste buds in the upper palate, tongue and floor of the oral cavity and tip of tongue.

2.1.3.2 Non-oral tissues

Numerous studies have demonstrated that taste receptors are present in a variety of extra-oral tissues and cells including the respiratory tract, liver, pancreas, kidney, testes, lungs, adipose tissue,

hypothalamus, endocrine glands, brain, gastrointestinal tract (GIT), enteroendocrine cell (EEC), smooth muscle cell, and STC-1 (Cheled-Shoval et al., 2015; Chen et al., 2006; Crowe et al., 2020; Roura et al., 2012; Rozengurt et al., 2006; Yamamoto and Ishimaru, 2013). RT-PCR analysis illustrated that chicken umami taste receptors are observed in the brain, oral, and gastrointestinal tissues (Yoshida et al., 2015). However, the functions of many of the non-oral taste receptors remain unclear. A recent study by Lee and Cohen (2015) demonstrated that sweet and bitter taste receptors expressed in the airway are involved in regulating innate immunity and host-pathogen interactions in mammals (Lee and Cohen, 2015). Bitter receptors found in human respiratory tract ciliated epithelial cells work as a sensory organ and mechanical force to propel harmful inhaled compounds out of the lungs (Shah et al., 2009). This result was supported by another study reporting that bitter substances such as denatonium benzoate, salicin, and phenylthiocarbamide (PTC) sensed by bitter receptors located in the epithelial cells increase intracellular Ca^{+} concentration causing the downstream response of accelerating ciliary beat frequency (CBF). As a result, this process increases toxins clearance, antimicrobial secretion, and bacterial killing (Workman et al., 2015). Therefore, it is necessary to study taste receptors in developing novel therapeutics to enhance innate immune defense and serve as antibiotic alternatives to treat respiratory diseases in the poultry industry. Unlike bitter receptors, T1Rs families recognize nutritional compounds and may have different functions in non-oral tissues. Cheled-Shoval et al. (2014 and 2015) have confirmed the expression of both chicken T1Rs and T2Rs subfamilies in the gut (Cheled-Shoval et al., 2014; Cheled-Shoval et al., 2015). The presence of umami taste receptors in a chicken's gut was also confirmed by Yoshida et al. (2015). Once these receptors have been triggered by corresponding tastants, these receptors will further initiate hormone production from the gut to modulate food

intake and other potential functions in non-oral tissues in chickens. This process remains to be elucidated.

2.1.4 Tastants / Ligands

The taste receptor ligands including agonist, antagonist, substrates, signaling lipids, and neurotransmitters which attach to target proteins to produce taste signals by the ligand-protein binding, are summarized in **Table 2.2**.

The basic tastes of sweet, bitter, umami, and kokumi receptors are large protein molecules which are composed of a ligand-binding domain (LBD) and cysteine-rich domain (CRD) followed by a transmembrane region (TM) connected to the intracellular domain (Nango et al., 2016). Sweet compounds such as sugar and artificial sweeteners; bitter tastants such as quinine and diphenidol; kokumi compounds such as calcium chloride ($CaCl_2$); and umami substances such as monosodium glutamate are mediated by transduction pathways through the ligand-binding domain (Nango et al., 2016). The lack of a T1R2 subunit in poultry makes it less capable of sensing sweet stimuli because of the absence of binding sites with ligands. The heterodimers of more than one binding site may be attributed to the synergistic effect of umami substances such as IMP, guanosine 5-monophosphate (GMP), and glutamate, and the ability of various chemically different compounds such as sweeteners, D-amino acids, and protein molecules to activate sweet taste (Depoortere, 2014). For example, both glutamate and IMP/GMP initiate the umami taste by binding to the N-terminal Venus flytrap domain of T1R1 (Li et al., 2002). The interactions of receptors and ligands are determined by the rate of binding affinity.

Table 2.2. Summary of tastants and corresponding taste modifying substances in poultry

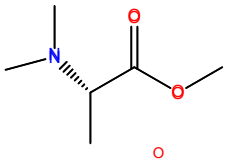
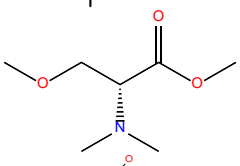
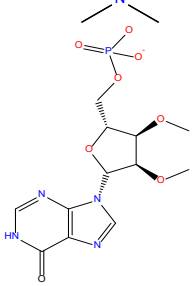
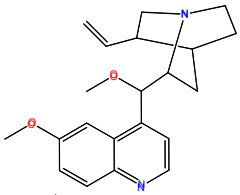
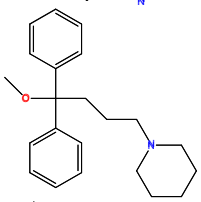
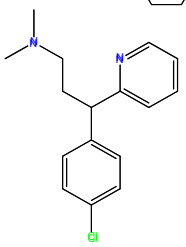
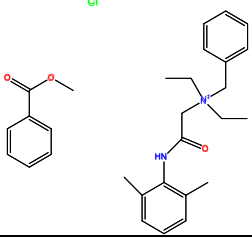
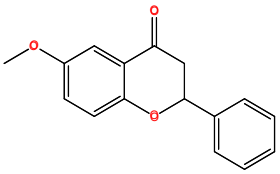
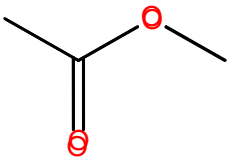
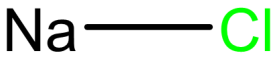
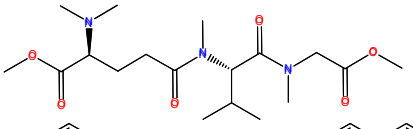
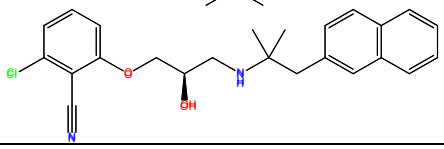
Taste	Substances	Chemical structures	References
Umami			
Agonists	L-alanine		(Baldwin et al., 2014)
	L-serine		(Baldwin et al., 2014)
Enhancers	Inosine 5'-monophosphate		(Chaudhari et al., 2009)
Bitter			
	Quinine		(Temussi et al., 2018)
	Diphenidol		(Temussi et al., 2018)
Agonists	Chlorpheniramine		(Temussi et al., 2018)
	Denatonium benzoate		(Jiang et al., 2020)

Table 2.2. Continued

Taste	Substances	Chemical structures	References
Bitter			
Antagonists	6-methoxyflavanone		(Dey et al., 2017)
Sour			
	Hydrochloric acid (HCL)		(Ganzevles and Koreze, 1987)
Agonists	Acetic acid		(Gentle, 1972)
Salty			
Agonists	Sodium chloride (NaCl)		(Roper, 2013)
Kokumi			
Agonists	γ-Glu-Val-Gly		(Ohsu et al., 2010)
Antagonists	NPS-2143		(Ohsu et al., 2010)

Umami and sweet receptors are low-affinity receptors with a millimolar range of EC_{50} for most of their ligands. This may be due to the evolutionary need of animals to detect foods high in nutrients (Depoortere, 2014). Computational methods showed that ggT2R1 is not only activated by quinine but also by three of its analogues including epiquinidine, ethylhydrocupreine, and quinidine (Di Pizio et al., 2017). Most bitter compounds activate all T2R families such as quinine, diphenidol, and chlorpheniramine (broadly "tuned"); however, others such as quinidine (selective ggT2R1 agonist) can only bind to one or two of the bitter receptors (Temussi et al., 2018). Intriguingly, chicken T2R7 can be activated by 17 bitter substances among 46 bitter compounds (Behrens et al.,

2014). In terms of kokumi, Tordoff (1996) showed that $CaCl_2$ can only initiate saltiness at a concentration level above 32 mM, indicating that $CaCl_2$ was not a component of calcium taste but a kokumi taste (Tordoff, 1996).

2.1.5 Umami taste receptors signaling pathway

Taste sense is responsible for animals detecting the nutrients and toxic food by chemosensing systems in lingual tissue and GIT. L-glutamate and some nucleotides such as IMP and GMP are umami ligands and further initiate downstream second-messenger signals and a series of effector systems to send the dietary information to the brain (Banik et al., 2018). Umami taste receptors share signalling pathways with bitter, sweet, and kokumi receptors by initiating G-protein coupled receptors (GPCRs) in cell membranes causing an increase of Ca^{+} in the cytoplasm which leads to cell depolarization and consequently the release of neurotransmitters (Torrallardona and Roura, 2009). Umami compounds are mediated by signaling transduction through the LBD which is part of the extracellular domain of T1R1/T1R3. Heterodimer T1R1/T1R3 receptors then cause the exchange of the GDP to GTP. Meanwhile, Gα subunits are dissociated from Gβγ together with binding to GTP, followed by initiating the downstream molecules phospholipase C (PLC)-β2, which in turn triggers the hydrolysis of phosphatidylinositol-1,4,5-biphosphate (IP_3) diacylglycerol (DAG) (**Figure 2.3**) (Behrens and Meyerhof, 2011; Toda et al., 2013). This process releases Ca^{2+} from the intracellular endoplasmic reticulum (ER), subsequently opens the transient receptor potential melastatin 5 (TRPM5) channel, finally causing sodium influx and depolarization of TRCs (Yamamoto and Ishimaru, 2013).

Taste receptors in the small intestine also send nutrient information to the brain through the vagal-brainstem-hypothalamic system (Depoortere, 2014). The gut-released endocrine peptides can

send satiation signals to the brain directly by bloodstream through the incomplete blood-brain barrier to the hypothalamus, or via the initiation of the vagus nerve (Depoortere, 2014). The chorda tympani plays an essential role in chicken taste perception because the chorda tympani nerve belongs to a branch of the facial nerve that rises from the taste buds in the tongue, which innervate the T1R1/T1R3 umami receptors in fungiform taste buds (Nelson et al., 2002).

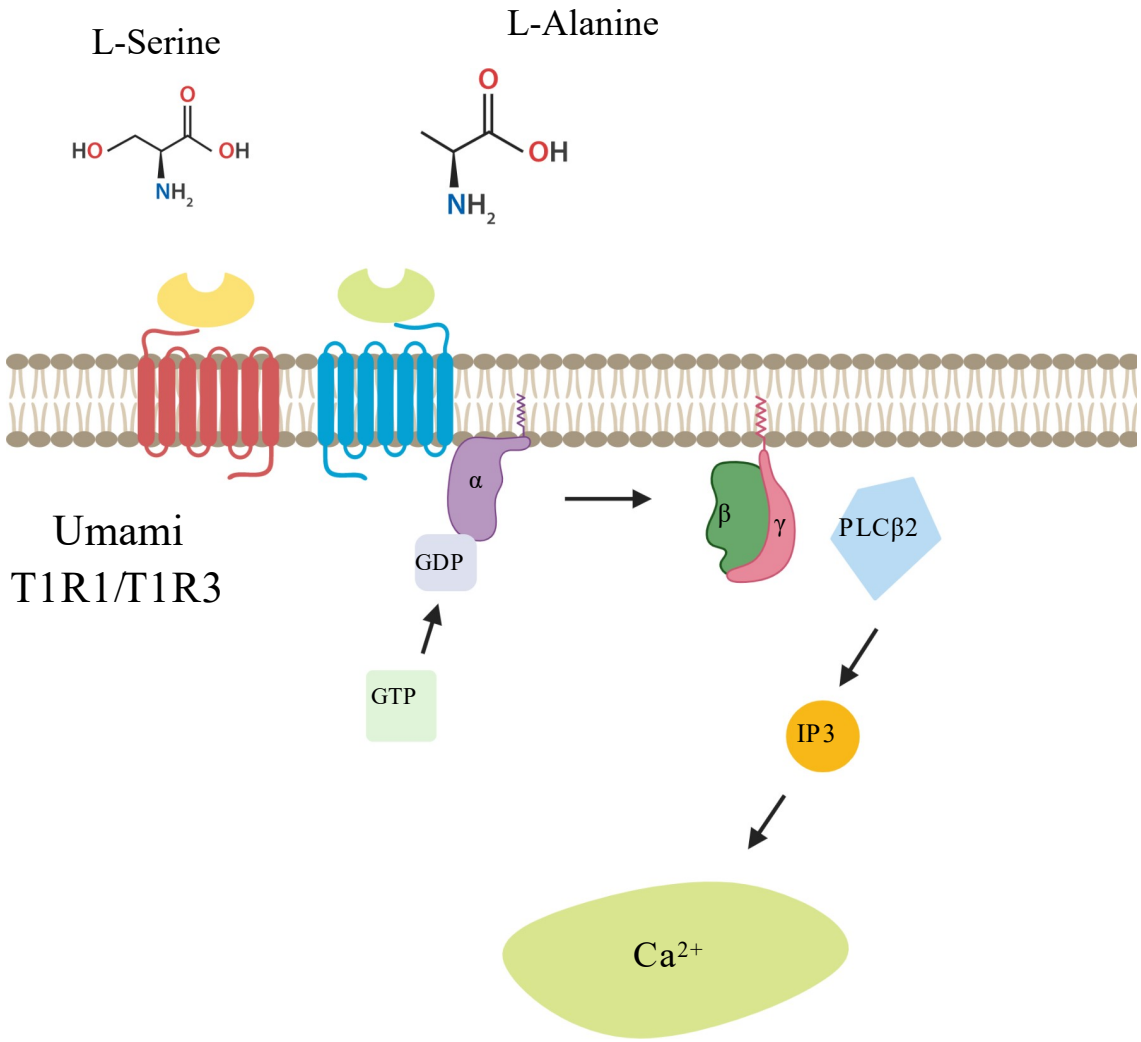


Figure 2.3. Schematic model of umami signaling pathway elicited by chicken umami stimuli

2.2 ROLE OF TASTE RECEPTORS IN THE REGULATION OF FEED INTAKE AND APPETITE

Taste receptors located in the oral tissue are associated with appetite, and those present as nutritional chemosensors in the gut play a crucial role in manipulating feed digestion (Sclafani and Ackroff, 2012), body metabolism (San Gabriel, 2015), feed utilization (Sclafani and Ackroff, 2012), and satiety effect (Scott, 2011) which provide the opportunity for farmers to improve the efficiency of the poultry industry (Yoshida et al., 2015). The expression of taste receptors of sweet, fatty acid, and umami in the gut may sense the luminal contents and modulate food intake by chemical signals and the endocrine system, for instance, through the secretion of incretin hormone from the intestine, glucagon/ insulin from the pancreas and leptin from adipose tissue (Wellendorph et al., 2010). The taste receptors and their sensed hormones in the gut system among different species are illustrated in **Table 2.3.**

Table 2.3. Summary of taste receptors and associated hormones in gastrointestinal tract in different species (Behrens and Meyerhof, 2019; Feher, 2012)

Taste	Taste receptors	Species	Ligands/stimulus	Hormone	EEC	Location	Reference
Sweet	T1R2/T1R3	Mouse	Aspartame, sucralose	Incretin, GLP-1, GIP, SGLT-1	L-cell K-cell	Duodenum	(Jang et al., 2007; Margolskee et al., 2007)
Umami	T1R1/T1R3	Human	Peptides, amino acids	CCK	I-cell	Duodenum	(Feher, 2012)
		Mouse	L-amino acids	CCK	STC-1 cell	Proximal small intestinal tissue	(Daly et al., 2013)
		Pig	Branched chain amino acids	CCK	STC-1 cell	Porcine jejunum	(Tian et al., 2019)
Bitter	T2R9	Human	Ofloxacin, procainamide, pirenzapine	GLP-1, GLP-2, PYY	L-cell	Distal small intestine	(Dotson et al., 2008)
	T2R138	Mouse	Cycloheximide	CCK, GLP-1	STC-1 cell	Small intestine	(Jeon et al., 2008)
	T2R108	Mouse	KDT501	GLP-1	STC-1 cell	Small intestine	(Kok et al., 2018)
Kokumi	CaSR	Human	Peptides, amino acids	CCK Gastrin	I-cell G-cell	Duodenum Antrum	(Feher, 2012)

Note. EEC, Enteroendocrine cell; GLP-1, Glucagon-like peptide 1; GLP-2, Glucagon-like peptide 2; GIP, Gastric inhibitory polypeptide; SGLT-1, Sodium/glucose cotransporter 1; CCK, Cholecystoknin; PPY, Peptide tyrosine tyrosine; STC-1, Intestinal secretin tumor cell line.

The hormones released from the gut act as appetite regulators in mammals. For example, ghrelin has been identified in the stomach and intestines of humans and rats as an orexigenic hormone to stimulate food intake. A number of studies have indicated that ghrelin workes as an anorexigenic peptide in chickens and can be detected in the gastrointestinal tract but not in the proventriculus, stomach, ileum and colon (Ahmed and Harvey, 2002; Honda et al., 2017). Ghrelin function as a stimulant of feed intake in avian species seems highly dependent on the dosage. In Japanese quail, for example, intraperitoneal administration (IP) of 0.5-1 nmol/bird of ghrelin increased food intake compared to 3nmol/bird (Honda et al., 2017). However, peripheral administration of 5-20 nmol/kg BW of ghrelin suppressed food intake in broiler chickens (Ocłoń and Pietras, 2011). Thus, the physiological function of ghrelin as a hunger hormone is different from breed to breed. On the other hand, gut hormones such as CCK are generally believed to be a satiety hormone in chickens (Honda et al., 2017; Reid and Dunn, 2018). A short-term study in chickens showed that both CCK and gastrin expression neither respond at 2.5 h nor 7.5 h post-feeding, contrary to the orexigenic action of a gut-released hormone in mammals (Honda et al., 2017). Therefore, chickens might develop a different physiological function of taste receptor-released gut hormones than mammals in the food intake and appetite regulatory system. These paradoxical results portend new technologies and experiments are required to better understand the role of taste receptor cells in regulating feed intake and appetite in chickens.

2.3 ROLE OF TASTE RECEPTORS IN GUT DEVELOPMENT AND GUT HEALTH

Understanding the nutrient-sensing mechanism of chicken taste receptors in the gut is necessary as the gut is the main location for feed digestion and absorption, which in turn, improves productivity. The recognition of nutrients by taste receptors along the GIT is of great potential

benefit to gut development and gut health through luminal or blood-borne direction and transmit signals that control the hormones released from the gut to maintain energy balance and GIT function (Depoortere, 2014). *In vivo* studies showed that dietary L-glutamine (L-Gln) supplementation has a beneficial effect on the barrier function of intestinal epithelial cells and meat quality, and enhances the immunity of chickens (Dai et al., 2009). Furthermore, L-Glu supplementation with submarginal crude protein and adequate essential amino acids can improve feed intake and growth in broiler chickens (Moran and Stilborn, 1996). However, a controversial study reported that Glu and Gln have no beneficial effect on growth performance (Shakeri et al., 2014). In a growth study, an improvement in weight gain, feed intake, and gain/feed ratio was observed when 1% L-arginine (Arg) was added to the corn-soybean meal diet with 4% excess L-phenylalanine (Phe) and L-tyrosine. T1R1/T1R3 not only sense luminal nutrients but also play a vital role in protein synthesis and cell growth (Wauson et al., 2012). Several studies have shown that in mammalian species, T1R1/T1R3 function as a direct sensor for L-amino acids to regulate the mammalian target of rapamycin complex 1 (MTORC1) via activating intracellular Ca^{2+} stimulation and extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Wauson et al., 2012; Zhou et al., 2016). The precise taste signaling of T1R1/T1R3 in the intestine is, however, still not clear. Studies performed in rodents suggest that bitter taste receptors sensing chemical signaling in the gut has been implicated in metabolic control, satiety regulation, and defensive functions (Dotson et al., 2008).

2.4 METHODS TO STUDY TASTE RECEPTORS

2.4.1 Molecular docking

Molecular docking is a computer-based application for screening ligands for the most compatible conformations of protein molecules and to evaluate the ligand-protein interactions based

on a three-dimensional (3D) structure which is broadly used in structural molecular biology and structure analysis drug design (Khan et al., 2017; Morris and Lim-Wilby, 2008). By using this method, the pose prediction between the protein of interest and ligands, virtual screening, and binding affinity are generated and analyzed for the binding properties of protein-ligands. There are two essential components of molecular docking: the 3D structure of target protein and small molecules binding with the target protein; and a procedure to predict the best poses and strength of protein-ligands interaction based on docking score rank (Balaji et al., 2013). Recently, more studies have used molecular docking to identify the interaction between taste compounds (e.g. sweeteners, bitter substances, and umami peptides) and taste receptors (Acevedo et al., 2016; Acevedo et al., 2018; Dang et al., 2019). Dang et al. (2019) used molecular docking to investigate the synergistic effect between MSG (umami peptide) and the taste receptor T1R1/T1R3. The results showed that the addition of MSG increases the size of the binding region which, in turn, caused more peptide binding into the T1R3 subunit (Dang et al., 2019). This suggests that molecular docking could be a vital application to screen ligands for target taste receptors in chickens.

2.4.2 Functional analyses

There has been rapid technological progress and advancement in investigating the function of cell membrane receptors, ranging from the reverse transcription-polymerase chain reaction (RT-PCR) to calcium imaging. Besides bioinformatic analysis, cell-based assays are required to support the scientific results of potential ligands for taste receptors, such as the ones from molecular docking. Currently, T1Rs and T2Rs families have been cloned and functionally characterized. Most of them are activated by carbohydrates, amino acids, proteolytic compounds or free fatty acids that are expressed in taste tissues (Wellendorph et al., 2010). Cell culture and molecular biology have been

widely used to study the signaling transduction and mechanisms of taste receptor cells. Calcium imaging, a common approach for detecting the functions of taste receptors to measure the intracellular calcium status when ligands are added and then cells express taste receptor genes, was conducted in numerous studies (Baldwin et al., 2014; Liu et al., 2018). Once the gene of interest is activated by ligands, the calcium imaging technique can be used to detect the fluorescent molecules that respond to intracellular Ca^{2+} modification. This technology is widely used in different cell types and neuron activities.

2.4.3 Dietary preferences: double choice studies

Unlike humans, animals cannot explain their taste preferences when consuming different feed ingredients. Therefore, behavioural studies are required for measuring innate dietary preferences in animals to study their taste perception and palatability. Among those, double choice studies are a commonly used method to determine the preferences for feed ingredients and potential taste substances. Balog and Millar (1989) used a double-choice test to detect the dietary preference between sweet, sour, salty, and bitter, and the results showed that broilers have a higher preference for aspartame and saccharin (sweet) followed by citric acid (sour), salt, and quinine (bitter) (Balog and Millar, 1989). Another study revealed that the umami taste has a synergic effect in chickens; it showed that a chicken response to umami ligands (e.g. L-alanine and L-serine) in various degrees depends on dosages (Yoshida et al., 2018). Another double choice study demostrated that chickens prefer consuming feed with the addition of both 0.5% MPG and 0.05% IMP rather than feed with either MPG or IMP on its own (Yoshida et al., 2015). Furthermore, a recent paper published from the same lab found that, in a 5-minute short-term behavior test, chickens prefer a solution containing both MSG and IMP (Yoshida et al., 2018). Using the same test, it was found that chicks prefer a

diet with an excess amount of amino acids compared to control balanced feed (Edmonds and Baker, 1987).

2.5 RESEARCH ADVANCES AND GAPS

Chickens have fewer taste receptor genes compared to other vertebrates. Because they lack the sweet taste receptor T1R2, chickens have less less sensitivity to sweeteners. In addition, bitter, sour, and salty taste sensations in chickens are highly correlated with age. Numerous recent studies have shown that CaSR receptors are mediated by kokumi substances; whether it is a potential taste receptor to recognized kokumi ligands is largely unclear. Understanding the role of taste receptors in gut health and disease is a new and emerging field which will provide better knowledge for developing novel compounds for antibiotic alternatives in the poultry industry (Depoortere, 2014). Recent research published by Reid and Dunn (2018) reported that taste receptors induced hormones such as ghrelin, gastrin, and CCK transmit satiety signals in humans and mammals, but the physiology functions of those hormones have the opposite effect in birds (Reid and Dunn, 2018). Therefore, more research regarding the regulatory genes and signaling pathways of feed intake in poultry are needed. Recently, more ligands associated with chicken taste receptors have been elucidated by using molecular docking and cell-assay based functional analyses. However, the specific function and potential ligands in precise concentration that can initiate chicken taste receptors are yet unknown. Chicken taste receptor cells are located in taste buds as well as in the intestines which might be involved in nutrient sensing. The molecular and cellular mechanisms of intestinal nutrient sensing are well studied in humans, mice, and rats, but more research is needed in poultry (Niknafs and Roura, 2018).

CHAPTER 3 HYPOTHESES AND OBJECTIVES

3.1 HYPOTHESES

The following hypotheses were tested in this thesis:

1. Umami taste receptors T1R1/T1R3 are expressed both in taste tissues and non-taste tissues in broiler chickens during embryonic and post-hatch development.

2. The chicken umami taste receptors T1R1/T1R3 have broad ligands specificity and ligands bind to the T1R1 extracellular domain.

3.2 OBJECTIVES

The overall objective was to investigate the expression and distribution of umami receptor T1R1/T1R3 in taste tissues and non-taste tissues with emphasis on the intestines in broiler chickens during embryonic and post-hatch development. Specific objectives were to:

1. Determine the expression and distribution of umami receptors T1R1/T1R3 in the digestive system in broiler chickens during late embryonic and post-hatch development.

2. Identify the binding locations of chicken T1R1/T1R3 receptors and their potential ligands.

CHAPTER 4 MANUSCRIPT

EXPRESSION OF UMAMI RECEPTORS (T1R1/T1R3) IN THE INTESTINE DURING EMBRYONIC AND POST-HATCH DEVELOPMENT IN BROILER CHICKENS

4.1 ABSTRACT

Food and water choices and consumption in animals is largely dependent on taste perception. Taste receptors in animals are present in taste tissues and non-taste tissues. In the gastrointestinal tract (GIT), these receptors function as chemosensors to detect chemical stimulus of luminal nutrients. In avian species as in the mammalian taste system, umami receptors T1R1/T1R3 mediate umami taste and these receptors can recognize protein breakdown products including amino acids and peptides. In the chicken genome, umami taste receptor genes have been found. However, the location of these taste receptors is not clear. In a preliminary study, we demonstrated that umami receptors T1R1/T1R3 are widely expressed in both the oral cavity and intestines of broiler chickens, and the jejunum has the highest expression level of umami receptors within the intestine in 35d broiler chickens. Gene expression of umami receptors T1R1/T1R3 in the intestine during embryonic and post-hatch development in chickens on the other hand is still unknown. The goal of this study was to investigate the gene expression of T1R1/T1R3 in the intestine during late embryonic and post-hatch development in broiler chickens. A total of 15 fertilized broiler eggs and 20 healthy broilers were used in this study. The jejunum was collected from 3 embryonic broiler chickens at embryonic day (E) 17, 18, 19, 20, and 21. Four chickens were randomly sacrificed at 1 week, 2 weeks, 3 weeks, 4 weeks, and 5 weeks, for collecting the duodenum, jejunum, ileum, and colon. We found the T1R1/T1R3 mRNA abundance was detected in the jejunum at all stages of late embryonic development. The jejunal T1R1/T1R3 mRNA abundance increased with age during late embryonic development in chickens. The expression of T1R1 in the duodenum and jejunum

changed quadratically ($P < 0.05$), while linear and quadratic patterns of changes ($P < 0.05$) were found in the colon during post-hatch development. These results demonstrated that chicken umami receptors T1R1/T1R3 are expressed in the intestine in the late embryonic stage and post-hatch stage and their level of expression may have been affected by the ages of embryonic and post-hatch development. However, the roles of chicken umami receptors T1R1/T1R3 in the intestine are still not clear. Further studies are needed to investigate the potential ligands of chicken umami receptors and cell signaling pathways activated by potential umami substances.

Key words: chicken umami receptors T1R1/T1R3, gene expression, intestine, gut chemosensing, chickens

4.2 INTRODUCTION

Young animals are the most vulnerable to disease, so antimicrobials are widely used in livestock production to fight diseases, maintain health and productivity. Global consumption of antimicrobials in food animal production was estimated at 63,151 tons in 2010 with an increasing trend and annual consumption of antimicrobials per kilogram of animal produced at 148 mg/kg and 172 mg/kg for chicken and pigs, respectively (Van Boeckel et al., 2015). These practices may have led to increased production of antimicrobial-resistant pathogens in both livestock and humans, posing a significant public health threat (Yang et al., 2015). Therefore, new strategies are needed to improve production and health. Gut chemosensing system has become an area of future investigation to further develop novel therapeutic strategies (Liu et al., 2013). The gut chemosensing system is considered archived by a network of G protein-coupled receptors (GPCR) that recognize nutrients, other chemicals, and microbes, and then initiates a cascade of signals to regulate nutrition, gut function, and metabolism (Liu et al., 2013). Identification of specific GPCR in the gut and their respective ligands can provide novel therapeutic targets for improving intestinal growth, barrier function, appetite and feed intake. Thus, understanding the relationship between gut chemosensing, integrity, and body metabolism in animals has emerged as an interesting research area in livestock animals. As part of the chemosensing system, taste receptors are GPCRs and play vital roles not only in distinguishing between beneficial nutrients and potentially toxic substances but also in nutrient sensing in intestines. However, more research on the molecular and cellular mechanisms of nutrient sensing by taste receptors in animals, especially poultry, is required to improve the production of chickens.

The gut operates not only as an organ to digest and absorb nutrients but also as a chemosensory system that, through cellular and molecular signaling cascading within the gut, the

brain, and other organs, mediates the orchestration of physiological and metabolic responses required to maintain homeostasis in mammals. There are several taste receptors including sweet, umami, bitter, salt, sour, fat, and kokumi in animals (Mace and Marshall, 2013; Roura et al., 2012; Tedo et al., 2011). Umami receptors have been identified and characterized in humans, mice and pigs (Tedo et al., 2011; Wauson et al., 2012; Xu et al., 2004). Unfortunately, our knowledge of umami receptors in poultry is very limited. A recent study demonstrated that bitter, sweet and umami taste receptors and downstream signaling effectors are expressed in the embryonic and growing chicken gastrointestinal tract. This is suggesting a very important role in the taste pathway contributing to the ability of chicken's gut to sense carbohydrates, amino acids and bitter compounds (Cheled-Shoval et al., 2015). Moreover, the demonstration of Gαgust and Gαtran expression in the chicken gastrointestinal tract provides evidence for taste receptor-mediated mucosal chemosensitivity (Cheled-Shoval et al., 2014).

Umami taste perception is mediated by an umami receptor (called taste 1 receptor member 1/member 3, T1R1/T1R3) belonging to class C of G-protein-coupled receptors (GPCR). T1R1/T1R3 receptors can recognize protein breakdown-related products, such as amino acids, peptides, and other chemicals. T1R1/T1R3 receptors are expressed both in taste tissue and non-taste tissues in mammals. To date, the molecular distribution of T1R1/T1R3 receptors and their gene expressions during embryonic and post-hatch development among intestinal segments are still largely unknown in poultry. Therefore, the objective of this study is to investigate the molecular distribution of umami receptor genes T1R1/T1R3 among different tissues in broiler chickens and preliminarily identify their expression patterns in the intestinal tissues during embryonic and post-hatch development.

4.3 MATERIALS AND METHODS

4.3.1 Animal management

The experiment protocol (#3675) was approved by the University of Guelph Animal Care Committee and the chickens were cared for according to guidelines of the Canadian Council on Animal Care (CCAC, 2009). All animal procedures were in compliance with animal welfare regulations and were reviewed and approved by the University of Manitoba Animal Care Committee (CCAC, 2009).

4.3.2 Tissue preparation

A total of 6 healthy broiler chickens age 35 days were obtained from the Arkell Poultry Research Station at the University of Guelph. The chickens were sacrificed using a CO_2 chamber. The upper palate, tongue, heart, lung, proventriculus (true stomach), ventriculus (gizzard), liver, kidney, duodenum, jejunum, ileum, cecum, and colon were cut into small pieces and stored in two 2-mL tubes, one filled with 1.2 mL RNAlater (ThermoFisher Scientific) and the other filled with 1.2 ml Allprotect Tissue Reagent (Qiagen) for real-time RT-PCR. All the samples were kept at -20°C for future analyses.

In order to study T1R1/T1R3 gene expression in jejunum during late embryonic development, a total of 15 fertilized Ross broiler eggs were obtained from a local hatchery (Maple Leaf Foods, New Hamburg, ON, Canada). Fertilized eggs were incubated under optimal conditions at the hatchery in Arkell Poultry Research Station, Guelph, ON, Canada. Briefly, 3 eggs were randomly euthanized by cervical dislocation at the embryonic days (E) 17, 18, 19, 20, and 21. Tissue samples of 1-3 cm jejunum segments were collected and placed into a 2 mL microcentrifuge tube with 1 mL of RNAlater. All samples were stored on ice and transferred immediately to the laboratory and kept at -80°C until RNA was extracted.

A total of 20 healthy Ross broiler chickens were used in the experiment to determine the T1R1/T1R3 gene expression level in intestinal segments during post-hatch development, and 4 chickens were randomly sacrificed by cervical dislocation at weeks 1, 2, 3, 4, and 5. Tissue samples of the duodenum, jejunum, ileum, and colon were collected and rapidly immersed in RNAlater and kept at 4°C for 24 hours. The samples were then transferred to -80°C and stored until RNA was extracted. The ingredient composition during post-hatch development is shown in **Table 4.1**. The details of the animal environment, housing, and management were described in our previous study (Choi et al., 2020).

Table 4.1. Nutrients composition of diets used in this study (g/kg, as feed basis).

Ingredients	0 to 14d	15 to 28d	28 to 35d
Corn ground	522.29	529.38	566.00
Soybean meal	305	261	225
Corn gluten meal, 60	35	35	35
Wheat shorts	25	30	30
Canola meal	25	30	30
Soya oil	22.6	43.8	45.8
DDGS, corn	20	30	30
Limestone	15	13	13
Vitamin premix broilers[1]	10	10	10
Biofos	9	7	5
Mineral premix broilers[2]	5	5	5
Methionine DL 99%	2.65	2.37	2.05
Lysine-HCL	2.25	2.46	2.31
Threonine	0.71	0.49	0.34
Xylanase 8000 G	0.2	0.2	0.2
Quantum blue 5000 G	0.3	0.3	0.3
Total	1000	1000	1000

Note. [1]Provided per kilogram of diet: retinyl acetate, 8250 IU; cholecalciferol 1000 IU; DL-alpha-tocopherol, 11 IU; cyanocobalamin, 0.012 mg; phylloquinone, 1.1 mg; niacin, 53 mg; choline, 1,020 mg; folacin, 0.75 mg; biotin, 0.25 mg; riboflavin, 5.5 mg.

[2]Provided per kilogram of diet: manganese, 55 mg; zinc, 50 mg; iron, 80 mg; copper, 5 mg; selenium, 0.1 mg; iodine, 0.36 mg; sodium, 1.6 g.

4.3.3 Bioinformatics analyses

The homology of T1R1/T1R3 mRNA and amino acid sequences between chicken and other species was collected from GenBank (https://www.ncbi.nlm.nih.gov/gene/428176) and analyzed by Clustal Omega which is a multiple sequence alignment online-tool (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al., 2011). The phylogenetic relationship of the T1R1/T1R3 amino acid sequences among Gallus gallus, Rattus norvegious, Homo sapiens, and Sus scrofa was further analyzed, and the phylogenetic tree was constructed by the maximum-likelihood model with 1,000 bootstrap replicates method in the MEGA 6 software (Tamura et al., 2011). A secondary structure model of chicken T1R1/T1R3 was constructed by Protter (http://wlab.ethz.ch/protter/start/). The three-dimensional structure models of T1R1 and T1R3 amino acid sequences were built by I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/).

4.3.4 RNA extraction and cDNA synthesis

Total RNA was isolated from 50 mg of tissue samples using an RNAqueous total RNA isolation kit (Ambion Inc., Foster City, CA) and treated by DNase I (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA quality was checked by using a 1% agarose gel electrophoresis stained with SYBR Green to represent the bands once separated (Invitrogen, Canada). The concentration and OD260:OD280 ratio of extracted RNA samples were measured using a Nanodrop UV-Vis spectrophotometer (Wilmington, DE) and the OD260:OD280 ratios were between 1.9 and 2.1. The RNA samples were stored at -80°C for further analysis.

4.3.5 Real-time RT-PCR analysis

A total of 1 μg RNA was used to synthesize the first-strand cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Mississauga, Canada) according to the manufacturer's instructions. Primers for the chicken T1R1, T1R3, Beta-actin (β-actin), and glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) genes were designed with Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and shown in **Table 4.2**. The primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Real-time RT-PCR was carried out using SYBR Green Supermix (Bio-Rad) on a CFX Connect Real-Time RT-PCR Detection System (Bio-Rad). 1 µL of cDNA was added to a total volume of 25 µL containing 12.5 µL SYBR Green mix, and 1 µM each of forward and reverse primers. We used the following conditions: denaturation 15 s at 95°C, annealing 15 s at 56°C, extension 30 s at 72°C, repeating 45 cycles. We used β-actin or GAPDH as the internal control to normalize the amount of starting RNA used in the real-time RT-PCR for all the samples. A melting curve program was conducted to confirm the specificity of each product and the size of the products was verified on SYBR-stained 2 % agarose gels in Tris acetate–EDTA buffer. Real-time RT-PCR analyses were performed in duplicate for each sample. The target gene expression was normalized with that of a selected reference gene and relative gene expression was determined using $R = 2^{(\mathrm{Ct}(\textit{reference})-\mathrm{Ct}(\mathrm{test}))}$ (Kleta et al., 2004). Threshold cycle (Ct) values were obtained at the cycle number at which the gene is amplified beyond the threshold of 30 fluorescence units. Real-time RT-PCR efficiencies were acquired by amplification of the dilution series of DNase-treated RNA according to formula $10^{(-1/\mathrm{slope})}$ (Pfaffl, 2001). The efficiencies of all primers used in this study were between 96-105%. Negative controls without cDNA were conducted along with each run, and each sample was analyzed in duplicate for each gene.

Table 4.2. Primers used for Real-time RT-PCR analyses

Chicken genes	Sequence (5'-3')	Length	Tm	Product size	GenBank accession no.
T1R1	F: ATCGGCTCTGTGATTGGCAT	20 bp	59.8°C	90 bp	KM091451
	R: GGCACGTTCCCTTGCATTTT	20 bp	59.9°C		
T1R3	F: CTCACCGAGTGCCTCTTCTG	20 bp	60.1°C	98 bp	KM091452
	R: CACATGAGCAGAACCTCCGT	20 bp	60.0°C		
β-actin	F: AATGGCTCCGGTATGTGCAA	20 bp	60.0°C	112 bp	NM_205518.1
	R: GGCCCATACCAACCATCACA	20 bp	59.9°C		
GAPDH	F: ACTGTCAAGGCTGAGAACGG	20 bp	59.9°C	99 bp	NM_204305.1
	R: CACCTGCATCTGCCCATTTG	20 bp	59.9°C		

T1R1: Taste 1 receptor member 1; T1R3: Taste 1 receptor member 3; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; F: Forward primer; R: Reverse primer; bp: base pair; Tm: Primer melting temperature.

4.3.6 Homology modelling of chicken T1R1/T1R3 extracellular domains

The extracellular domain of chicken T1R1/T1R3 structure was built using the SWISS-MODEL (https://swissmodel.expasy.org) server. The target amino acid sequences of chicken T1R1 and T1R3 were obtained from the UniProtKB Proteomes portal (https://www.uniprot.org/proteomes/) in FASTA format. The SWISS-MODEL generated multiple chicken T1R1/T1R3 templates from the PDB database and ranked from highest to lowest according to the structure quality of the models. The best model was selected based on templates 5x2m.1

(Crystal structure of the medaka fish taste receptor T1r2a-T1r3 ligand-binding domains in complex with L-glutamine) with a shared amino acid sequence identity of 37.05%.

4.3.7 Ligand docking to T1R1/T1R3 in Schrödinger's Glide

The 3D model of chicken T1R1/T1R3 was pre-processed and energy minimized by the Protein Preparation Wizard (Maestro, Schrödinger, LLC, New York, NY, 2020) to create an accurate, reliable, and all-atom protein model with proper bond order and formal charges. The grid area for docking pocket was generated in the Venus fly trap (VFT) domain of the receptor which is set as the centroid of workspace ligand for ligand docking.

A total of 15 compounds including L-amino acids, sugars, peptides, and nucleotides were docked to the chicken T1R1/T1R3 model as shown in **Table 4.4**. The 2D chemical structure of 15 compounds was downloaded from the PubChem database and optimized using Ligprep Module using Schrödinger (Maestro, Schrödinger, LLC, New York, NY, 2020). The optimized compounds were docked into the chicken T1R1/T1R3 protein structure using Glide (SP & XP) module. Final scoring and favourable interactions were carried out on the ligand poses by the GlideScore function.

4.3.8 Statistical analyses

All the data received from real-time PCR were shown as means ± SEM and analyzed by SAS 9.4 (the SAS Institute, Cary, NC). Significant differences of individual comparisons were statistically analyzed by one-way ANOVA with Tukey's multiple comparison test. Orthogonal polynomial contrasts were used to establish the linear, quadratic, and cubic effects of T1R1/T1R3 gene expression level of intestine segments at different ages. All figures were made by GraphPad Prism 8 (GraphPad Software, La Jolla, USA). Statistical significance was considered if $P < 0.05$.

4.4 RESULTS

4.4.1 Phylogenetic analyses

The full chicken T1R1 and T1R3 mRNA coding region sequence shares 54.63%, 55.76%, and 55.12% identity with mice, humans, and pigs, respectively. The amino acid sequence homology between chicken T1R1 and that of mice, humans, pigs ranges from 40.56% to 43.06%, and the chicken T1R3 amino acid sequence identity is between 45.99% and 47.63% compared with the three other species. As shown in **Fig. 4.1**, the phylogenetic trees of T1R1 and T1R3 revealed that all amino acid sequences for both T1R1 and T1R3 could be clustered into two groups: the mammalian group and the avian group. Chicken T1R1 and T1R3 belong to the avian group, while mouse, human, pig T1R1/T1R3 are located in the mammalian group. The evolutionary relationships reveal the chicken was furthest away compared with the other vertebrate species.

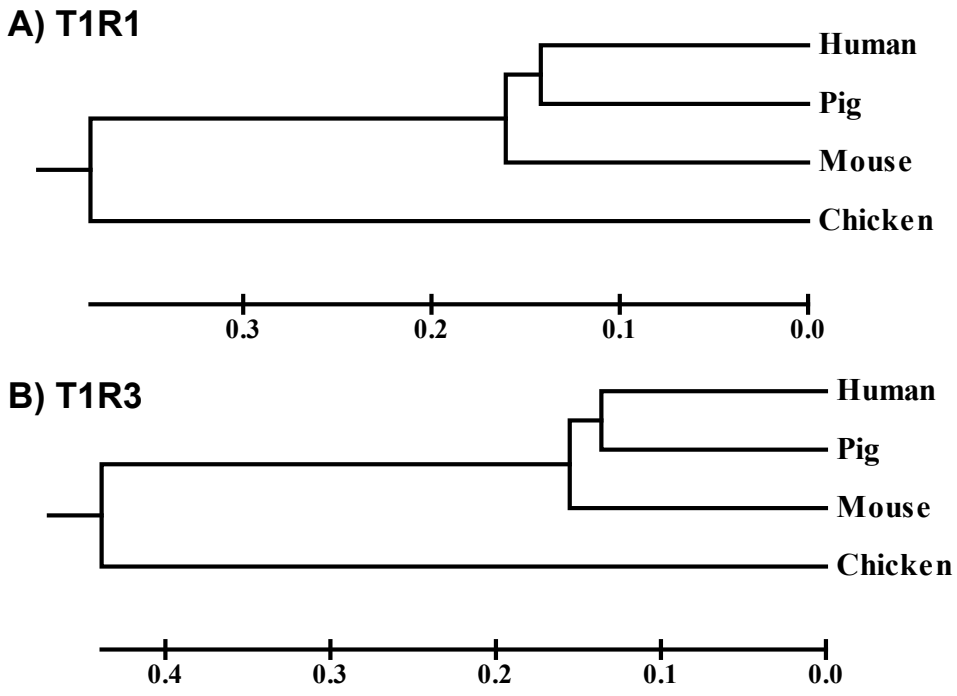


Figure 4.1. The phylogenetic trees of T1R1 and T1R3 amino acid sequences among chicken and three other animal species.

4.4.2 Structure features of chicken T1R1 and T1R3 protein

According to the secondary structural model of the chicken T1R1/T1R3 amino acid sequence built by Protter (**Fig. 4.2**), the chicken T1R1 protein is comprised of 836 amino acids (aa) including a 20 aa N-terminal signal peptide (1-20), a large extracellular domain (ECD) of 541 aa (21-562), seven transmembrane domain (TMD) containing 243 aa (562-805) with three extracellular loops, three intracellular loops, and an intracellular domain (ICD) comprised of 31 aa (805-836). Chicken T1R3 protein contains 834 aa in total, including 16 aa N-terminal signal peptides (1-16), a 547 aa ECD (563-16), a 242 aa TMD (805-563), and one 29 aa ICD (834-805).

According to the three-dimensional (3D) protein homology model of chicken T1R1 and T1R3 ECD which was constructed by using the SWISS-MODEL (Fig. 3), the ECD for both T1R1 and T1R3 consists of two main parts; the VFT domain which is formed by two lobes (lobe 1 and lobe 2), and the cysteine-enriched (CYS) domain. As for the 3D protein structure models of chicken T1R1 and T1R3, seven transmembrane helixes were shown with different colors (except green) linked with N-terminal sequences outside the cell membrane and C-terminal sequences inside the cell membrane (**Fig. 4.3**).

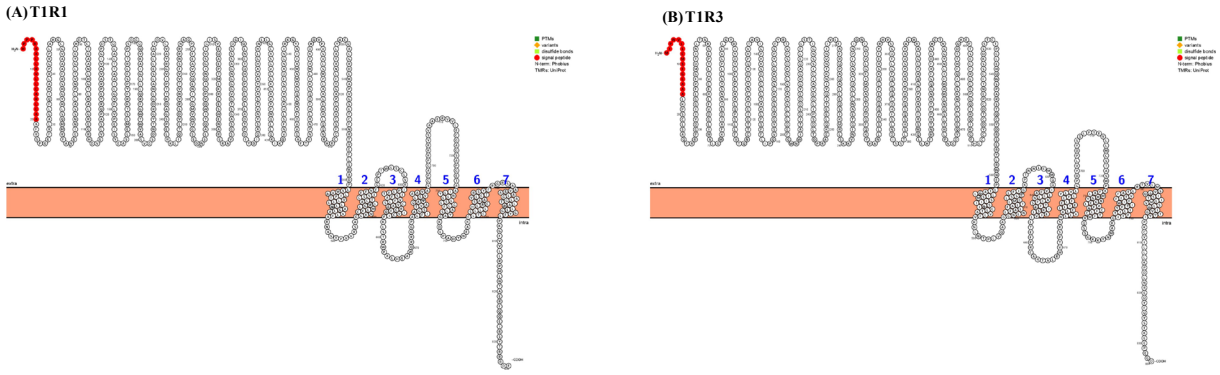


Figure 4.2. Secondary structure model of chicken T1R1 and T1R3 amino acid sequence

The protein of T1R1 is comprised of 836 amino acids and the first 20 amino acids form a signal peptide. The secondary structure of chicken T1R3 is similar to chicken T1R1. The protein of T1R3 is comprised of 834 amino acids and the first 16 amino acids form a signal peptide.

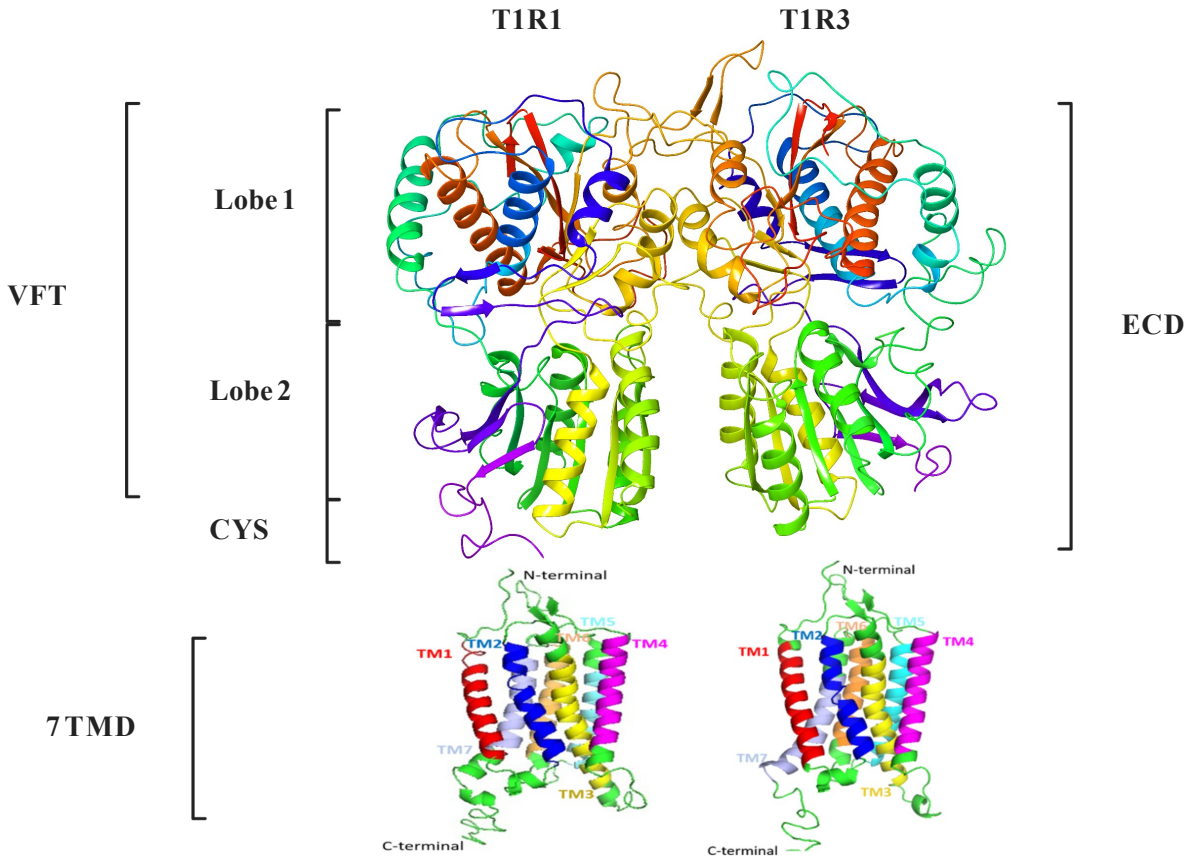


Figure 4.3. Three-dimensional structure models of chicken T1R1/T1R3 amino acid sequences

Seven transmembrane helixes are shown, each with a different color (excluding green). On each of helix, the amino acids with the color differing from green are in the transmembrane regions.

4.4.3 Relative T1R1/T1R3 mRNA abundance

As showed in **Fig. 4.4**, the gene expression levels of chicken T1R1/T1R3 were detected in many different tissues including the upper palate, stomach, tongue, heart, lung, kidney, gizzard, duodenum, jejunum, ileum, and colon of Ross broiler chickens. The mRNA abundance of T1R1/T1R3 was higher in the duodenum and jejunum ($P < 0.05$) than in the other tissues. The jejunum had the highest gene expression level in chicken intestine among duodenum, ileum, and colon ($P < 0.05$). However, no significant difference of T1R1 gene expression was observed in the

upper palate, stomach, tongue, heart, lung, kidney, or gizzard ($P > 0.05$). Among all 11 tissues, the T1R3 mRNA abundance showed no significant difference except in the jejunum.

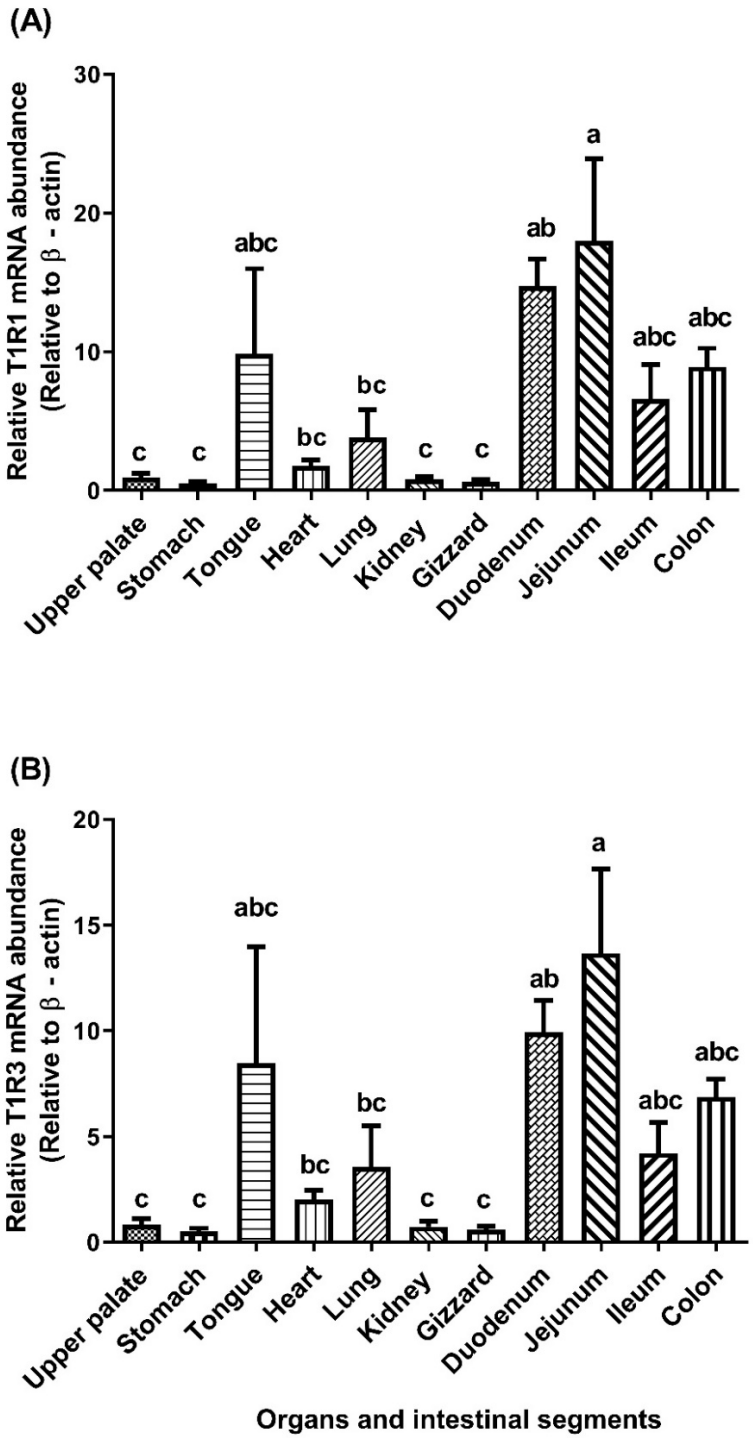


Figure 4.4. Relative T1R1/T1R3 mRNA abundance in the upper palate, stomach, tongue, heart, lung, kidney, gizzard, duodenum, jejunum, ileum, and colon of Ross broiler chickens ($P < 0.05$)

4.4.4 Relative T1R1/T1R3 mRNA abundance during embryonic development

The gene expression levels of T1R1/T1R3 in the jejunum for chickens in the embryonic development stage were analyzed using real-time RT-PCR. As shown in **Fig. 4.5A**, the mRNA abundance of T1R1 showed a linear and quadratic pattern ($P < 0.05$) in embryonic development. The mRNA abundance at the age of E20 was significantly higher than at the ages of E17 and E18 ($P < 0.05$). However, there was no significant difference in the T1R1 mRNA abundance in the jejunum at E19 and E21 ($P > 0.05$). As shown in **Fig. 4.5B**, the T1R3 mRNA abundance in the jejunum changed in a linear and cubic pattern ($P < 0.05$) during the late embryonic development stage. The T1R3 expressed in the jejunum was significantly higher at the age of E20 and E21 ($P < 0.05$). In addition, the T1R3 mRNA abundance was significantly higher at the age of E19 than at the ages of E18 ($P < 0.05$). However, the mRNA expression level of T1R3 showed no significant differences in the jejunum on E17 and E19 ($P > 0.05$).

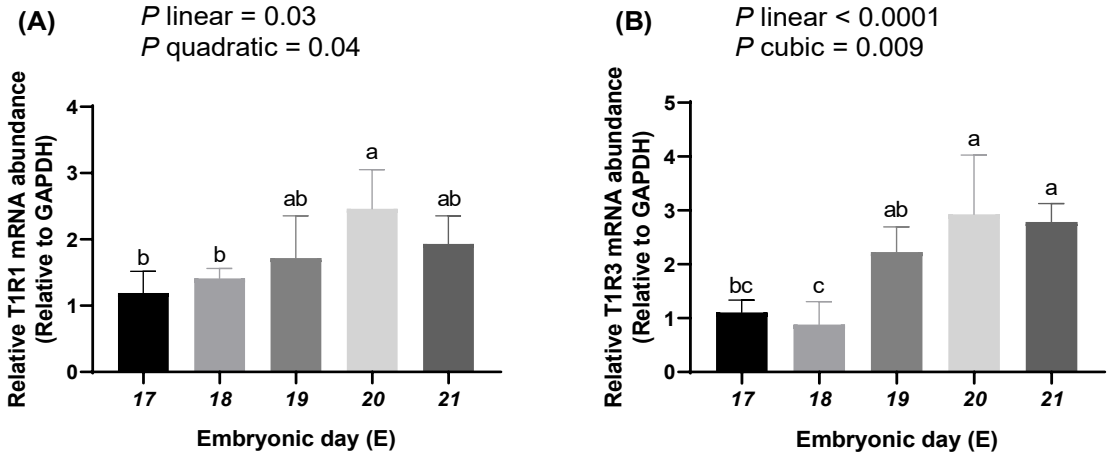


Figure 4.5 (A and B). Relative T1R1/T1R3 mRNA abundance during embryonic development

Results were normalized with GAPDH and data are presented as mean ± SEM, n = 3. There were significant differences between bars with different letters ($P < 0.05$).

4.4.5 Relative T1R1/T1R3 mRNA abundance during post-hatch development

The expression of T1R1/T1R3 genes in the duodenum, jejunum, ileum, and colon of chicks at the different ages was measured by real-time RT-PCR, and the abundance of T1R1/T1R3 mRNA relative to the first week was calculated. As shown in **Table 4.3**, the gene expression of T1R1 in the duodenum and jejunum showed a similar trend during development. Expression was initially high in the first week, gradually decreased until a significantly lower expression was reached in the third week, and then increased, although this increase in the duodenum was not significant ($P > 0.05$). Gene expression in the ileum and colon followed a similar trend. The expression was initially high in the first week, decreased significantly in the second week, remained low in the third week, and then gradually increased from the fourth week to the fifth week.

The expression of T1R3 in the duodenum, ileum, and colon did not change significantly during development. However, the expression in the jejunum gradually increased, and a significant difference was observed in the fifth week ($P > 0.05$).

Table 4.3. Relative mRNA abundance of umami taste receptors in the intestinal segments during the post-hatch development at day 7, 14, 21, 28, and 35 of broiler chickens

Items[1]	Age (d)					SEM[2]
	7	14	21	28	35	
T1R1						
Duodenum[3]	1.23[a]	0.72[ab]	0.49[b]	0.87[ab]	0.88[ab]	0.08
Jejunum	1.01[a]	0.94[ab]	0.58[b]	0.90[a]	0.76[ab]	0.05
Ileum[3]	1.74[a]	0.98[c]	0.82[c]	1.62[bc]	1.48[ab]	0.12
Colon[3,4]	1.75[a]	0.97[b]	1.09[b]	1.53[a]	1.70[a]	0.08
T1R3						
Duodenum	0.47	0.52	0.33	0.47	0.54	0.04
Jejunum	0.31[b]	0.53[ab]	0.48[ab]	0.50[ab]	0.62[a]	0.05
Ileum	0.95	0.67	0.81	0.60	1.06	0.06
Colon	0.85	0.74	0.82	0.83	0.95	0.03

[a,b,c] Comparison of significant differences in each row were established at $P < 0.05$ among different ages (n=4).

[1]T1R1 = Taste 1 receptor member 1, T1R3 = Taste 1 receptor member 3

[2]SEM: pooled standard error of the mean

[3]Quadratic effects of gene expression in different intestinal segments among the age groups

[4]Linear effects of gene expression in different intestinal segments among the age groups

4.4.6 Molecular model building of chicken T1R1/T1R3 and ligand docking

A 3D structure of chicken T1R1/T1R3 extracellular domain was built by the SWISS-MODEL server. The extracellular domain of T1R1 consists of 561 amino acids, and the T1R3 receptor consists of 562 amino acids. A total of 15 potential chicken umami agonists including L-amino acids, peptides, nucleotides, and sugars that were summarized in **Table 4.4** were docked into the chicken T1R1/T1R3 model.

Table 4.4. Final scoring of 15 ligands binded with chicken T1R1/T1R3 protein

Number	Ligands	Docking score	Glide gscore	Glide emodel
1	GMP	-7.105	-7.197	-82.244
2	IMP	-6.417	-6.744	-77.126
3	Lys-gly	-5.543	-5.635	-68.714
4	Saccharin	-5.400	-5.400	-37.878
5	L-proline	-5.326	-5.327	-28.848
6	Fructose	-4.942	-4.942	-36.516
7	L-valine	-4.886	-4.886	-22.385
8	Aspartame	-4.67	-4.689	-41.024
9	L-arginine	-4.493	-4.493	-28.934
10	L-cystine	-4.412	-4.426	-24.370
11	Gln-glu	-4.351	-4.482	-60.852
12	L-serine	-4.131	-4.131	-21.950
13	MSG	-3.971	-3.971	-29.531
14	S807	-3.770	-3.770	-42.852
15	L-alanine	-3.450	-3.450	-16.459

Note. GMP, Guanosine monophosphate; IMP, Inosine 5'-monophosphate; Lys-gly, L-lysine and glycine residues; Gln-glu, L-glutamine and L-glutamic acid residues; MSG, L-monosodium glutamate

The 3D molecular structure of ligand docking was described in section 4.3.7. The docking results showed that the binding pocket in the chicken T1R1/T1R3 model is located on the extracellular domain between lobe 1 and lobe 2 in the T1R1 receptor (**Fig 4.6A**). All 15 compounds were docked into the same binding pocket, which was shown in **Fig 4.6B**.

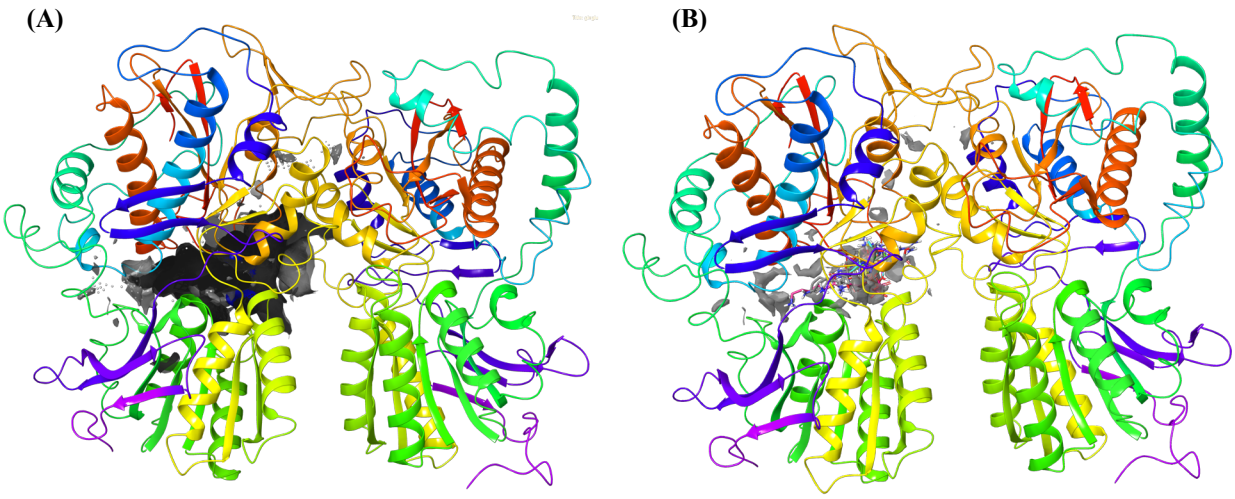


Figure 4.6 (A and B). Molecular model of chicken T1R1/T1R3 extracellular domain

(A) The binding pocket of T1R1/T1R3 is located on the VFT domain between lobe 1 and lobe 2 in the T1R1 receptor. (B) Overlay of T1R1/T1R3 bound to 15 potential ligands within the binding pocket.

4.4.7 Ligand docking of GMP and L-alanine into T1R1/T1R3

Both GMP and L-alanine were bound within the same binding pocket in the extracellular domain of the T1R1 receptor (**Fig 4.7A** and **Fig 4.8A**). The ligand docking results of T1R1/T1R3 bound to GMP and L-alanine suggest that ARG265, CYX93, and GLU95 on chain A of the T1R1 receptor make direct contact with both compounds (**Fig 4.7B** and **Fig 4.8B**). In the GMP docking complex, the oxygens on the phosphate group of GMP make hydrogen bonds with the residues of ASN205, ASN266, ASN263, and a salt bridge with ARG265, while the ARG265 also form a hydrogen bond with the oxygen atom of ribose. In addition, GLU95 and CYX93 form hydrogen bonds with two ribose hydroxyl groups (**Fig 4.7C**). In the L-alanine docking complex, the amino acid residues of GLN35 form a hydrogen bond with oxygen on the carboxylate group; the

ammonium makes hydrogen bonds with GLU95 and CYX93. Meanwhile, residues of GLU95 and ARG265 make salt bridges with an amino group and oxygen ion, respectively (**Fig 4.8C**).

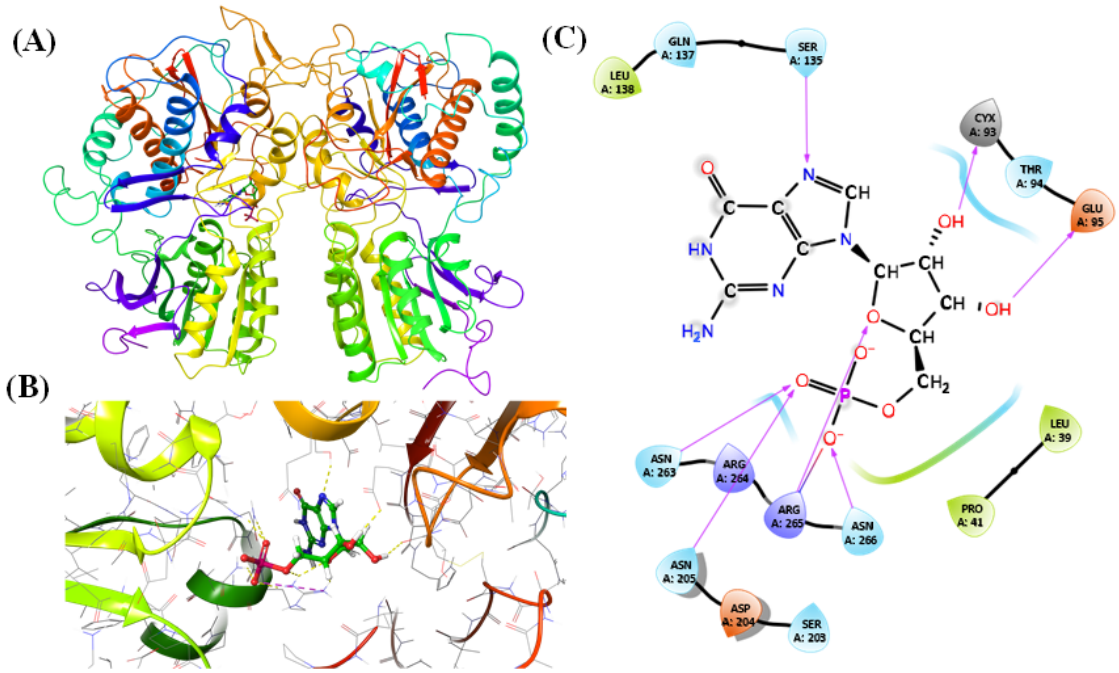


Figure 4.7. Molecular model of chicken T1R1/T1R3 bound to guanosine 5-monophosphate (GMP)

(A) The binding pocket of chicken T1R1/T1R3 bound to GMP. (B) Insert panels showing T1R1/T1R3 bound to GMP and the amino acid residuals within the 4Å region. (C) A 2D structure syncs with a 3D structure of insert panels of T1R1/T1R3 bound to GMP within the ligand-binding pocket.

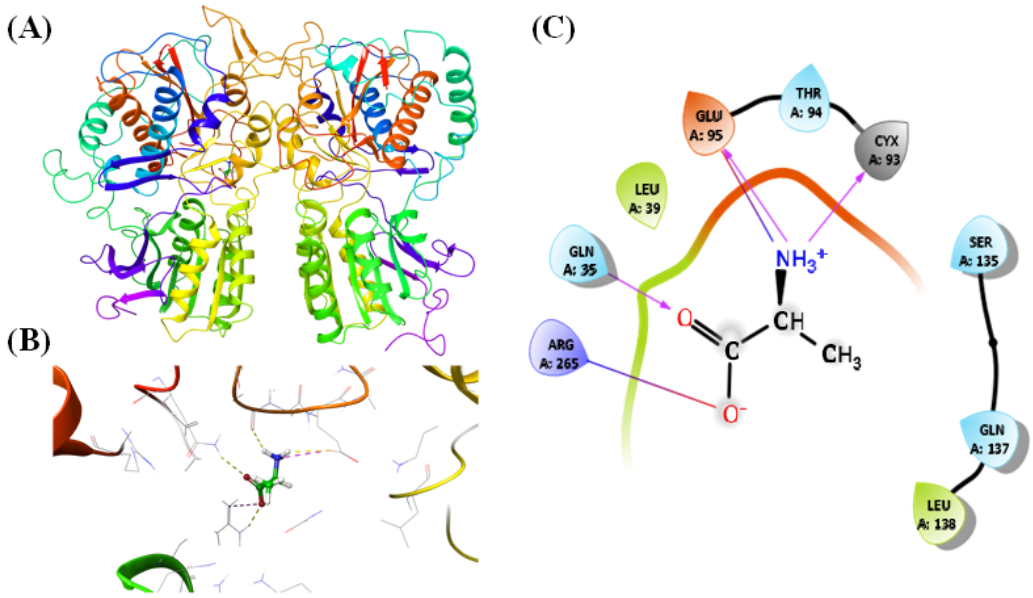


Figure 4.8. Molecular model of chicken T1R1/T1R3 bound to L-alanine

(A) The binding pocket of chicken T1R1/T1R3 bound to L-alanine. (B) Insert panels showing T1R1/T1R3 bound to L-alanine and the amino acid residuals within the 4Å region. (C) A 2D structure syncs with a 3D structure of insert panels of T1R1/T1R3 bound to L-alanine within the ligand-binding pocket.

4.5 DISCUSSION

GIT is a major sensory organ in detecting and recognizing nutrient and non-nutrient signals that control nutrient transporter expression, gut motility, nutrient digestion and absorption, and gut hormonal excretion to mediate feed consumption and nutrient utilization (Cheled-Shoval et al., 2015; Depoortere, 2014; Oliver et al., 2007; Margolskee et al., 2007; Murovets et al., 2015; Roura et al., 2011). Umami taste receptors T1R1/T1R3 expressed in the oral tissue are also expressed in the gut and play a role in detecting nutritional signals, such as L-amino acids, peptides, and other chemicals

but not D-amino acid enantiomers (Nelson et al., 2002; Roura et al., 2011). In chickens, the oral cavity is the major organ for taste sensation, but recent studies have confirmed that both T1R1 and T1R3 are present in GIT in embryonic and post-hatch chickens (Cheled-Shoval et al., 2015; Yoshida et al., 2015). This suggests that T1R1/T1R3 receptors may play an important role in sensing amino acids and other chemical signals in the chicken gut. The molecular distribution and functional characterization of T1R1/T1R3 have been widely studied in humans and mice (Brand, 2000; Di Pizio et al., 2019; Kendig et al., 2014; Kurihara and Kashiwayanagi, 2000; Liu et al., 2018; Yasumatsu et al., 2009). However, the relative mRNA abundance of umami receptors during embryonic development and post-hatch development is scarce (Byerly et al., 2010; Kudo et al., 2010). In the current study, we demonstrated the basic structural features of the chicken T1R1/T1R3 gene. Also, the tissue distribution of T1R1/T1R3 mRNA in the intestinal segments during embryonic development and post-hatch development in chickens was investigated.

Like humans, chicken umami taste is sensed by the heterodimeric receptors T1R1/T1R3 which belong to the class C GPCRs. According to alignment results of the T1R1/T1R3 amino acid sequences of chicken with other animal species, it was shown that chicken T1R1/T1R3 amino acids had the highest homology with the common pheasant (*Phasianus colchicus*), followed by the Japanese quail (*Coturnix japonica*), human, rat and pig. This is in agreement with the findings of other studies (Gloriam et al., 2007). Chicken T1R1/T1R3 share a conserved structure of GPCRs with other species, such as humans, mice, and fish (Acevedo and Temussi, 2019; Behrens et al., 2011; Kiuchi et al., 2006). Our results also indicated that T1R1/T1R3 were highly conserved in the mammalian and avian genomes. Both chicken T1R1 and T1R3 proteins contain 7 transmembrane helixes, 3 extracellular loops, 3 intracellular loops, and an extracellular (ECD) and intracellular domain (ICD) with various amino acid residues such as the T1R1 and T1R3 proteins in other species.

Moreover, similar 3D structural models were observed between human and chicken T1R1/T1R3, comprised of N-terminal signal peptides; an ECD containing 2 lobes of VFT domain; and linked with a CYS domain; a seven-helix transmembrane domain (7 TMD); and an ICD (Acevedo and Temussi, 2019; Behrens et al., 2011; Raliou et al., 2011).

However, a higher identity of protein sequence was usually found among mammalian species (Diaz et al., 1997). A low identity of the chicken T1R1/T1R3 genes was found among mammalian species; this identity was higher among other avian species. Thus, chicken T1R1/T1R3 genes may have different functions than T1R1/T1R3 genes of mammalian species (Gloriam et al., 2007). It is reasonable to speculate that the chicken T1R1/T1R3 may respond to exogenous stimuli differently than in mammalian species. Therefore, more research is required to characterize the functions of chicken T1R1 and T1R3 receptors.

The mRNA abundance of T1R1/T1R3 was detected in the upper palate, tongue, heart, lung, proventriculus (true stomach), ventriculus (gizzard), liver, kidney, duodenum, jejunum, ileum, cecum, and colon. This was consistent with the results of other studies (Cheled-Shoval et al., 2015; Yoshida et al., 2015). In mammals, T1R1/T1R3 have also been reported in various tissues and cells including testis (Kiuchi et al., 2006), hypothalamus (Byerly et al., 2010), enterocytes, paneth cells (Sbarbati and Osculati, 2005), cardiac myoblasts (Wauson et al., 2012), and gastric smooth muscle cells (Crowe et al., 2020). The function of taste receptors in the gut are not for taste sensation but as a sensor to detect chemical stimulus of luminal nutrients. In the current study, T1R1/T1R3 mRNA expressed in the lower GIT tissues (duodenum, jejunum, ileum and colon) were higher than those in the non-taste organs; these results were similar to those observed in other mammalian species (Daly et al., 2013). Results from our study were in agreement with Cheled-Shoval et al. (2015). These authors reported that T1R1/T1R3 were expressed in both gustatory tissues and extra-

gustatory tissues including upper GIT and lower GIT in E19 and 21 d chickens. They reported that T1R1/T1R3 expression in the colon was significantly higher than in the duodenum in 21 d chickens (Cheled-Shoval et al., 2015). In our study, however, no significant difference of T1R1/T1R3 expression was observed among lower GIT tissues in 35 d broiler chickens. This may be due to the different age and breed of chickens being used for gene expression analysis between the two studies. The intestinal segments are the main digestion and nutrient absorption site where the detection of luminal nutrients (such as L-amino acids) it is of higher significance than in other non-digestive tissues in chickens. This hypothesis is supported by other studies. The luminal nutrients sensed by TR-expressing epithelial cells in the gut induce the secretion of neuropeptide hormones and carry chemical signals by activating the vagal nerve to the brain regulating nutrient utilization, gut motility, appetite, and body metabolism (Crowe et al., 2020; Depoortere, 2014; Farré and Tack, 2013; Kaji and Kaunitz, 2017; Kokrashvili et al., 2009; Raybould, 2010; Roura et al., 2012; Sclafani and Ackroff, 2012; Steensels and Depoortere, 2018; Vella and Camilleri, 2017).

We further investigated the gene expression level of umami taste receptors at late embryonic development in the jejunum, since T1R1/T1R3 mRNA abundance was expressed at much higher levels in the jejunum than in the other non-taste organs tested. We found that T1R1/T1R3 were expressed at all stages of late embryonic development. Interestingly, the mRNA expression in the small intestine of pre-hatch stage chicks during development showed the same trend as pre-hatch taste bud development in chickens (Ganchrow and Ganchrow, 1987). Similar to taste bud development in embryonic chickens, both T1R1 and T1R3 expressed in chicken jejunum tissues during embryonic development changed with age (Rajapaksha et al., 2016). Furthermore, higher expression was observed at a late embryonic age, indicating that T1R1/T1R3 genes may play an important function in late embryogenesis for detecting nourishment from the yolk through the yolk

sac membrane (Li et al., 2008). More studies are needed to elucidate the growth-related expression of taste receptors and their function in animal gut development.

The expression of T1R1/T1R3 in the intestinal segments in broiler chickens during post-hatch development was also investigated in the current study. The T1R1 mRNA abundance in the duodenum and ileum changed in a quadratic pattern during post-hatch development. The lowest expression of T1R1/T1R3 in the duodenum and jejunum was observed at week 3. Similarly, the expression of T1R1 mRNA abundance in the ileum and colon at week 1 was significantly higher than the expression in weeks 2 and 3. The expression of T1R1/T1R3 at all stages of pre-hatch and post-hatch development in intestinal segments suggests the involvement of signaling pathways in sensing amino acids and other chemical compounds in chicken GIT. To date, little is known about the chicken gut development pattern of mRNA abundance for many chemosensing receptors and transporters including those that are well studied in mammals. The gene expression of T1R1/T1R3 in the post-hatch stage during gut development in our study showed a similar trend with the expression of luminal chemosensors in chicken gut development. Some nutritional transporters such as peptide transporter 1 (PepT1), excitatory amino acid transporter 3 (EAAT3), sodium glucose cotransporters 1 (SGLT1), and fructose transporter GLUT5 in the chicken small intestine were highly expressed at the age of 7 d and decreased by the age of 14 d; all of them showed linear and quadratic patterns of expression during development (Mott et al., 2008). This abrupt change of nutritional transporters at early post-hatch development was also observed by Miska and Fetterer (Miska and Fetterer, 2019). In a pigeon study by Zhang et al. (2017), a significant decline of amino acid transporters mRNA abundance including alanine-serine-cysteine-1 (Asc-1), cationic amino acid transporter 1 (CAT-1), and Na^+-dependent transporter for proline and hydroxyproline (IMINO) were observed at 14 d in the post-hatch pigeon (Zhang et al., 2017). The probable explanation of

the high expression of these luminal chemosensors at the early post-hatch period is due to metabolic efficiency or the requirement for early nutrients in rapid growth Ross broiler chickens right after hatch (Mott et al., 2008). Similarly, if the expression level of T1R1/T1R3 genes correlates with a greater amino acid sensation and absorption, then the greater expression in early post-hatch and its decline observed at weeks 2 and 3 may be due to the nutritional and energy requirement changes at different life stages (Drewnowski, 2000; Mott et al., 2008). Thus, a better understanding of the umami taste receptor in the chicken gut during development is needed in order to facilitate the advancement in gut health and growth performance through dietary formulation by studying the expression of T1R1/T1R3 genes.

In the current study, the gene expression of T1R1 was highly expressed and showed linear and quadratic patterns in the colon during post-hatch development. These results are in line with the findings of Cheled-Shoval et al. (2015) which showed that the gene expression level of T1R1 was highest in the tongue and colon compared to T1R3 in those tissues (Cheled-Shoval et al., 2015). In mammals, amino acids and other chemical signals in the colon sensed by T1R1/T1R3 cause secretion of calcitonin gene-related peptide (CGRP) and further initiate the peristaltic reflex and colonic propulsion (Kendig et al., 2014). A recent study by Crowe et al. (2020) showed that the peristaltic reflex was inhibited in T1R1 knockout mice, suggesting a potential role of T1R1/T1R3 agonists in regulating gut motility (Crowe et al., 2020).

Although the T1R3 mRNA abundance in the duodenum, ileum, and colon during the post-hatch development did not change significantly, the expression of T1R3 in the jejunum increased with age after hatch. This suggests that solo T1R3 plays a limiting role in luminal sensing, particularly monosaccharide taste sensation during the post-hatch stage (Mace et al., 2015). In mammals, the T1R3 subunit is responsible for the luminal monosaccharide sensor to regulate the

secretion of dependent glucose transporter isoform 1 (SGLT1), and the greatest expression of SGLT1 was found in the jejunum tissues in chickens (Margolskee et al., 2007; Mott et al., 2008). Sulistiyanto et al. (1999) also found that the mRNA level of SGLT1 was low at the newly post-hatch stage and gradually increased with age, which is in agreement with our study (Sulistiyanto et al., 1999). The low expression of the T1R3 at early post-hatch may be because of the hydrophobic yolk residuals in the lumen and lower amounts of lumen sodium than at hatching (Noy and Sklan, 1999). The gene expression of the T1R3 subunit in the jejunum in our study showed to be somewhat different from that of the T1R1 subunit, suggesting the T1R3 subunit alone has other functions such as carbohydrate sensing, which differs from umami taste receptor functions in gut chemosensing systems.

In the present study, the gene expression level of T1R1 was more highly expressed than T1R3 in the intestinal segments in chickens. The VFT domain of the T1R1 subunit plays an important role in sensing umami ligands compared to the T1R3 subunit because the T1R1 subunit contains the most binding sites for umami substances such as L-glutamate (L-Glu) and inosine-5'-monophosphate (IMP) (Belloir et al., 2017). Nuemket et al. (2017) demonstrated that the T1R3 subunit has an auxiliary role with T1R1; this may explain the synergistic response to umami stimuli (Nuemket et al., 2017; Yoshida et al., 2015). The current findings may be useful for paving the way in novel therapeutic and new feed additive development for chickens based on their gut expression level during different growth stages.

The molecular modelling results indicated that the ggT1R1/T1R3 broadly tuned by various chemical stimuli are controlled by the amino acid residues in the VFT domain of T1R1. The extracellular domain of T1R1/T1R3 molecular structures of humans and mice were built by previous studies based on mGluRs and have been identified using x-ray crystallography (Kunishima

et al., 2000). However, there is no amino acid structure available for ggT1R1/T1R3. In this study, we crystallized the structure of chicken T1R1/T1R3 and then docked into 15 potential umami agonists to identify the receptor-ligand interaction. As shown in **Fig 4.6A**, the ligand-binding pocket site of the umami receptors T1R1/T1R3 in chicken is located in the hinge region between the VFT domain of T1R1 which is similar with other species (Toda et al., 2013). The docking results indicated that all potential umami compounds were bound into the same binding pocket, suggesting that they may play a similar role for umami receptor activation in chickens.

Results from ligand screening analysis showed that 3 amino acid residues are essential for chicken umami taste to bind with GMP and L-alanine: GLU95, CYX93, and ARG265. We also predicted the free energy of ggT1R1/T1R3 binding with different compounds in **Table 4.4**. Intriguingly, GMP and IMP had the higher docking scores and L-alanine and L-serine had a lower docking score when docked into the chicken T1R1/T1R3 structure. Based on previous studies, IMP and GMP are umami ligands that strongly enhanced the umami taste specifically in humans (Zhang et al., 2008). Other vertebrate species such as mice, fish, and chickens showed a stronger umami taste response to L-amino acids rather than IMP and GMP (Morais, 2017). Our results showed that chicken T1R1/T1R3 can be broadly tuned by L-amino acid, peptides, ribonucleotides and other chemical compounds. We also found that few sweeteners can bind into chicken T1R1, in contrast to a cell-based assay, where it was shown that chicken T1R1/T1R3 can not be activated by sugars due to a lack of the T1R2 gene necessary for sweet taste sensation (Baldwin et al., 2014). The possible reasons could be 1) molecular docking scores are prediction tools for protein-ligand binding energy which may not be accurate; 2) currently, there is no crystal structure for chicken T1R1/T1R3 and the template we used to build chicken umami taste receptors, that is the fish T1R2/T1R3 receptor (sweet receptor), shares a low amino acid sequence identity with chicken

T1R1/T1R3. Therefore, computational approaches are not enough for ligand screening, and further *in vitro* and *in vivo* studies are needed to fully understand the mechanisms of chicken umami taste receptors.

Ligands docking is based on computational algorithms for docking and scoring involved in predicting binding modes over a wide range of cocrystallized structures. With Glide, the best-docked structure is made using a model energy score., i.e., Emodel, that combines the energy-grid score and the binding affinity predicted by GlidScore (Friesner et al., 2004; Hevener et al., 2009). Significant errors in binding mode predictions are found in some test cases (Hevener et al., 2009; Warren et al., 2006). Although Glide accuracy is better than alternative packages such as DOCK, FlexX, GOLD, and Surflex, there are still errors associated with initial screening, energy minimization and binding affinity prediction (Friesner et al., 2004). These authors reported that the root-mean-square deviations (rmsd) (Å) of atomic positions were smallest for various ligands compared to values obtained with the other packages. Still, the rmsd values ranged from 0.45 to 2.83 Å for various ligands when the most accurate scoring results were obtained. The fact that the rmsd was higher than zero and in some cases above 10 Å is indicative that Glide, as with the other packages, does not always result in the most accurate answer (Friesner et al., 2004). Although a perfect pose match may not be possible, meaning a rmsd close to zero Å, and the cut-off values selected may vary among studies (Warren et al., 2006), one would expect a better match as rmsd drops and the best results when rmsd is closer to zero. The calculation of the rmsd values in comparing the cocrystallized structure with the docking results could be used as a validation technique for the molecular docking as it is a measure of the docking accuracy. However, in our study, we did not calculate the rmsd as the cocrystallized structure of the receptor, and some of the ligands used in docking are not known. As GlideScoring simulates binding free energy, more

negative values represent tighter binders. Higher negative values were obtained with GMP than with the other ligands tested, and the lowest negative score was obtained with L-alanine, suggesting that a looser binding was obtained with this ligand compared to the other ligands tested. This simulated result agrees with the finding that GMP in chickens is an umami ligand that strongly enhances the umami taste sensation as it does in humans (Zhang et al., 2008). The Emodel, on the other hand, has a more significant weighing of the force field components, that is electrostatic and van der Waals energies, making it more suitable for comparing conformers but not as suitable for comparing chemically distinct species. This means that the Emodel alone should not be used as a base of comparison of GMP and L-alanine scoring, as these two molecules are chemically distinct. In this case, Glide uses the Emodel to pick the best pose of a ligand, and the score obtained by *GlideScore* through the ranking of the best poses against one another should be followed, meaning that these two scores should be analyzed together. When taken into account together, scores were higher for GMP and lower for L-alanine compared to all other ligands tested. Whether the L-alanine docking score from the Emodel were low compared to the GMP score and that of other ligands is not clear. However, chickens, like fish, show a stronger umami taste response to L-amino acids than they do to IMP and GMP (Morais, 2017).

A key issue affecting the ligand docking accuracy is how closely one of the conformations generated by Glide matches the correct cocrystallized conformation (Friesner et al., 2004). The fact that the crystallized structure of T1R1/T1R3 of chickens is not known and the structure of T1R2/T1R3 of fish was used instead may be an oversimplification and an error source on the docking modelling used in the present study. Characterization of the chicken T1R1/T1R3 crystallized structure would help produce better docking modelling results so researchers could use

the closest conformation to the correct actual cocrystallized conformation of T1R1/T1R3 of chickens.

In conclusion, we found that chicken T1R1/T1R3 share a low identity of coding mRNA sequence, protein sequence, and similar secondary and 3D structure among mammalian species. Furthermore, chicken T1R1/T1R3 are widely expressed in different tissues in broiler chickens, and jejunum has a higher level compared to other intestinal segments. We also demonstrated that the mRNA abundance of chicken T1R1/T1R3 changed with age during the embryonic development in the jejunum. In addition, the gene expression of T1R1 changed significantly with age, especially at weeks 2 and 3 in the intestinal segments during post-hatch development. The chicken T1R1/T1R3 binding pocket is located in a hinge region between two lobes of the VFT domain, and broadly tuned by various ligands.

CHAPTER 5 GENERAL DISCUSSION AND CONCLUSIONS

5.1 GENERAL DISCUSSION

The attention given to the potential applications of taste receptors has increased in the animal industry because nutrients sensed by taste receptors in the oral cavity and GIT influence not only behavioral but also physiological responses. For example, taste receptors increase appetite and growth performance in animals (Hartley et al., 2019). Understanding the role of taste receptors in chickens is a potential way to cope with antibiotic abuse in feed and improve growth performance and gut health. Among the five basic tastes, the umami taste was first discovered by a Japanese scientist in the late 1900s, and later, the heteromeric receptors T1R1 and T1R3 were identified as umami taste receptors (Kurihara, 2015). Recent advancements in methodology, such as molecular biology and behavior testing have shown that T1R1/T1R3 receptors are responsible for detecting free L-glutamate (glutamic acid) and MSG in humans (Roura et al., 2011). These ligands showed subordinately in chickens compared to other L-amino acids, such as L-alanine and L-serine (Baldwin et al., 2014).

In the current study, the expression of T1R1/T1R3 was localized both in the oral cavity and non-oral tissues, which indicated that umami taste receptors may have other functions in different organs other than taste sensation by taste buds in the tongue. We further confirmed that umami taste receptors were expressed in the chicken intestinal tissues and during the late embryonic and post-hatch development, suggesting that T1R1/T1R3 may act as nutrient-sensors in the intestinal tissues to sense nutrient stimulus and regulate gut motility, digestion, and absorption (Yoshida et al., 2018). The intestinal epithelium acts as a barrier to toxins, antigens, and bacteria while allowing the absorption of nutrients, electrolytes, and water (Tan, 2019). However, when the gut is faced with homeostatic challenges and imbalances, it may cause gut disorders and result in gut inflammation

with detrimental effects on animal performance and health (Hui et al., 2020). Increasingly, research has demonstrated that amino acids play a key role in preventing and attenuating gut inflammation (He et al., 2018). Therefore, further studies are needed to elucidate the specific role of T1R1/T1R3 in chicken intestinal tissues. Furthermore, the ligands that activate T1R1/T1R3 receptors in the intestine may be different than the ligands that more strongly activate this receptor in oral tissues. It is possible that the ligands are the same but they are needed in different concentrations for the receptors in these two different tissues. This needs further investigation.

The molecular docking prediction results showed that chicken T1R1/T1R3 may be broadly tuned by L-amino acids, peptides, and nucleotides. The activation of the chicken T1R1/T1R3 gene by the predicted 15 ligands, requires, however, further confirmation by *in vitro* and/or *in vivo* studies. Using calcium-sensitive photoprotein reporter assays, Baldwin et al. (2014) reported *in vitro* activation of the chicken umami receptors T1R1/T1R3 by L-alanine and L-serine measured in heterologous cells (Baldwin et al., 2014). The same approach could be used to test the receptor response to other ligands. This approach would not only help us to identify the stronger ligands in the activation of chicken T1R1/T1R3 receptors but would also allow us to validate the ligand docking simulation results. Although root-mean-square deviations are a measure of ligand docking accuracy, they are statistical and empirical in nature and this accuracy needs to be backed up by other validation methods such as the *in vitro* assays indicated above. Furthermore, *in vivo* studies could further validate ligand docking and help to identify important ligands or tastants in T1R1/T1R3 activation in chickens. One way to accomplish this would be to use a two alternative forced-choice solution consumption method where different concentrations of a ligand would be investigated as opposed to the cconsumption of water alone by chickens (Shovel et al., 2017). These authors found that chicks were less sensitive to sucrose and L-monosodium glutamate with

threshold concentrations 1000 higher than ligands such as quinine (bitter) which was below 1mM and similar to humans. This study was conducted with 4 d old chicks and for a short period of time of 24 h. Also, these authors did not test the bird's responses to L-amino acids. It would be interesting to evaluate if similar results would be obtained with chickens of older ages and with shorter trial durations with various L-amino acids. Baldwin et al. (2014), in a behavioral assay involving hummingbirds, recorded the behaviour of these birds when presented with test stimuli for 15 min; they recorded preferential behaviour of birds to ligands such as sucrose, glucose and fructose over water and L-amino acids. It is very interesting that although hummingbirds, like chicken, lack the essential subunit T1R2, which is the only known vertebrate sweet receptor, they positively and strongly sensed sugars which were not observed in chickens. Chicken T1R1/T1R3, on the other hand, failed to detect carbohydrates at any concentration level tested and instead recognized alanine and serine. The reason for this difference between these two birds is not clear, but Baldwin et al. (2014) proposed that a change in the umami taste receptor function of T1R1/T1R3 enabled hummingbirds to perceive and use sugars as opposed to chickens. This needs further investigation. It is important to keep in mind that the study by Baldwin et al. (2014) investigated only the receptors from oral tissues. It could be speculated that the function of taste receptors in the oral vs. non-oral tissues may have different responses to the same ligand probably due to the specific function of the receptors in the oral tissues versus non-oral tissues like the receptors in the gut. Therefore, the activation of T1R1/T1R3 may be tissue-specific, a theory which needs further investigation.

5.2 GENERAL CONCLUSION

In conclusion, umami taste receptors T1R1/T1R3 are expressed in the oral tissues and non-oral tissues in broiler chickens. In addition, chicken taste umami receptors T1R1/T1R3 are

expressed in the intestinal tissues in the late embryonic and post-hatch stage and their expression level changes with age. Chicken T1R1/T1R3 may be broadly tuned by L-amino acid, peptides, and nucleotides based on the protein-docking prediction tool. However, the activation of the chicken T1R1/T1R3 genes by 15 predicted ligands requires further confirmation with *in vitro* and/or *in vivo* studies.

CHAPTER 6 FUTURE DIRECTIONS

Further research is needed to investigate:

1. The potential ligands of chicken umami receptors and cell signalling pathways elicited by potential umami compounds.

2. The crystalline structures of chicken T1R1/T1R3 extracellular ligand-binding domains, the recognition domain of the chicken T1R1/T1R3 heterodimer, the bond to different amino acids, peptides, and the potential ligands of chicken T1R1/T1R3 genes.

3. The effects of agonists on the activation of T1R1/T1R3 in broiler chickens by either *in vitro* or *in vivo* studies.

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