Doctoral Dissertation(Shinshu University)

Effects of dietary nutrients on structure and function of the chicken intestinal mucosa

（ニワトリ腸管粘膜の構造及び機能に対する食餌中栄養素の影響）

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Abstract

Effects of dietary nutrients on structure and function of the chicken intestinal mucosa

Histological and functional features of the intestinal mucosa solely determine the digestive and absorptive ability of avian species. The inner epithelial lining of the gut is folded into fingerlike structures called villi that are lined by the single columnar cell layer. This epithelial cell layer is crucial for the proper digestion and absorption of nutrients. Microvilli are tiny fingerlike projections on the apical surface of the epithelial enterocytes that increase mucosal surface area and reside in various nutrient receptors, channels, and transporters for adequate digestion and absorption. Enteroendocrine cells are located in the epithelial cell layer and synthesize and secrete various hormones to perform a variety of physiological and biological functions in the gut. Glucagon-like peptides (GLPs) and neurotensin (NT) are peptide hormones secreted to ingested nutrients and participate in intestinotrophic and various physiological functions in the intestine. In this dissertation, various morphological, immunohistochemical, and *in situ* hybridization techniques were employed to demonstrate the impact of dietary nutrients on structural characteristics and functional diversity in the chicken intestinal mucosa, elucidating the following points. (1) Low dietary CHO induces structural alterations in the enterocyte microvilli. This study used control and three experimental groups (50%, 25% and 0% CHO of control diet). The findings demonstrated that loosely packed and fragmenting microvilli were predominant in the lower CHO groups. There was a gap between neighbor microvilli in the lower CHO groups, particularly in the 0% group. In addition, microvilli length, width and density

were significantly lower in lower CHO groups (P<0.05). Moreover, frequencies of GLP-2-immunoreactive and proglucagon mRNA-expressing cells showed a decreasing trend with declining CHO levels in the diets. Therefore, this study indicated that the CHO levels have a role in the structural integrity of chicken ileum enterocyte microvilli. (2) Dietary protein level influences NT-immunoreactive cells in the distal ileum. The control (CP18%) and three experimental groups (CP 9%, CP 4.5% and CP 0%) were set in this study. Low protein ingestion provoked morphological modification of NT-immunoreactive cells and showed a significant decreasing trend of cells frequencies (P<0.05). This study showed that dietary protein is an essential activator of NT synthesis and release in the distal ileum. (3) Dietary carbohydrate stimulates the colocalization pattern of GLP-1 with NT in the ileum. This study has three groups: a control group and two treatment groups (Low-CHO and CHO-free). The colocalized cells showed a long cytoplasmic process in the control group. The frequency of colocalization of GLP-1 with NT-immunoreactive cells was significantly increased in the control group (P<0.05). These findings suggest that dietary CHO has an effect on the functional capacities of the intestinal mucosa. In conclusion, the present dissertation study demonstrated that dietary CHO could influence ileal mucosal formation. Moreover, the dietary CHO and CP positively affect the activation of intestinal L and N cells. Therefore, it can be stated that dietary nutrients are a potent stimulator for altering intestinal mucosal structure and function in chicken, as studied in this dissertation.

DEDICATED TO MY BELOVED WIFE AND RESPECTED SUPERVISOR Kohzy HIRAMATSU, Ph.D.

Contents

Chapter I

General Introduction

General Introduction

Gastrointestinal (GI) system in animals is a major nutritional consumer, with excessive cell turnover necessitating the consumption of large quantities of macronutrients. As a consequence, an increase in the proportionate size of the GI tract would raise the nutritional requirement of the gut and decrease the feed efficiency of the bird. In order for avian GI tract efficiency to be maximized, it is essential that its functional anatomical and histological features would be maintained (De Verdal *et al.,* 2010). The small intestine, in comparison to other sections of the GI tract, is a significant location for digestion and absorption of nutrients that are readily accessible. In the bird, the histological characteristics of the mucosa are linked with the digestive and absorptive capacities of the small intestine. Villus is a critical histological component in this section (Ragaa and Korany, 2016). The epithelium of the small intestine is twisted into villi lined with single-layer columner cells covered apically by tiny fingerlike structures known as microvilli and collectively recognized as a brush border. This improves the small intestinal available cell surface area for absorption by about 600 times, leading to greater capacity for nutrient absorption (Wijtten *et al.,* 2012). The fuctional abilities of the intestinal mucosa depend on the capacity of individual enterocytes. The number of villi and the number and architecture of microvilli on the surface of individual enterocyte define the maximal contact area of the epithelium. It has been shown that the quantity and size of microvilli vary among different species (Smith *et. al*., 1984; Ferraris *et al*., 1989). In addition, the length and structure of enterocyte microvilli in mammals are also affected by a variety of factors, including dietary nutrients. Previous studies demonstrated that the development of microvilli on the surface of enterocyte affected by the level of various nutrients in isocaloric diets (King *et al.,*1983; Dauncey *et al*., 1983).

In the GI tract, there are around 20 different kinds of enteroendocrine cells (EECs). L cell and N cell are two of the them in this group. Glucagon-like peptide (GLP)-1 and -2 are released from intestinal L cells in response to ingested food. This two peptide hormones are synthesized in the intestine from a common precursor protein called proglucagon through a process of post-translational proteolytic cleavage and other enzymatic modifications (Lovshin and Drucker, 2000; Burrin *et al*., 2003; Drucker, 2005; Drucker, 2006; Richards and McMurtry, 2009). In the small intestine, these two peptide hormones exert a variety of physiological and biological functions. GLP-1 is a 36-amino acid peptide classified as an incretin hormone that is released in a nutrient-dependent manner to lead to distinct physiological functions. These functions include enhancement of glucose-dependent secretion of insulin, suppression of glucagon release and a rise in pancreatic β cell proliferation (Fridolf *et al.,* 1991; Nauck, 1998; Drucker, 2006), a reduction in food intake (Balkan, 2000), delaying of gastric emptying (Nauck, 1998), lowering intestinal motility (Tolessa *et al.,* 1998), and promoting the growth of the intestine (Kissow *et al*., 2012). GLP-2 is a 33-amino acid peptide that is well-known for its trophic properties in the gut (Thulesen, 2004). This meal-induced peptide hormone has a variety of actions on the GI tract. This hormone aids in the growth of the intestine by boosting epithelial cell proliferation while reducing apoptosis (Guan *et al*., 2006). It induces intestinal epithelial cells to grow more slender and longer, with longer microvilli visible on the enterocyte's luminal surface (Benjamin *et al*., 2000). Additionally, it inhibits gastric acid release, decreases gastric motility, regulates food intake, and accelerates the transfer of hexose into plasma membrane vesicles (Cheeseman and Tsang 1996; Wojdemann *et al*., 1999; Brubaker, 2006).

Neurotensin (NT) is a 13 amino acid peptides, isolated for the first time from bovine hypothalamic extracts (Carraway and Leeman, 1973), secreted fron intestinal N cells. The precursor protein for this hormone has 169-170 amino acids and is an extremely conserved polypeptide in rats, dogs, and cows, as well as other mammals (Dobner *et al.,* 1987; Kislauskis *et al*., 1988). This hormone has a variety of physiological functions including in the GI tract, such as regulating gastric and intestinal motility (Thor and Rosell, 1986), inducing pancreatic and biliary secretion (Gui *et al*., 2001), promoting mucosal growth (Evers, 2006), and increasing capillary permeability (Harper *et al*., 1984).

The distribution of EECs throughout the GI tract is linked with the particular function of the respective areas of the GI tract. Furthermore, the morphological characteristics of the EECs are associated with the functions of these cells. The GLP-1- and GLP-2-immunoreactive cells are extensively detected in the small intestine and colon of mammals with a few exceptions in the upper duodenum and other regions of the chicken intestine (Hiramatsu *et al.,* 2003, 2005; Monir *et al.,* 2013; Latorre *et al.,* 2016). The presence of NT-immunoreactive cells was found throughout the intestine (Atoji *et al.,* 1994). Researchers demonstrated that L and N cells have a long cytoplasmic process and that their apical surface is covered with microvilli (Helmstaeder *et al*., 1977; Sundler *et al.,* 1982; Nishimura *et al.,* 2013). According to Hiramatsu (2020), these kinds of EECs are classified as "open-type" cells since they may receive intraluminal nutrients as signals and release their hormones in endocrine and paracrine manner (Gribble and Reimann, 2016). Numerous studies have shown that specific nutrients may have an impact on the hormone synthesis and secretion from their respective cells in the GI tract. Several *in vivo* and *in vitro* studies demonstrated that carbohydrate, starch metabolites, glucose, fat, fatty acid and protein stimulated the secretion of GLPs and NT from L-cells and N-cells,

respectively, in mammals (Rosell and Rökaeus, 1979; Go and Demol, 1981; Holst Pedersen *et al*., 1988; Elliott *et al*., 1993; Herrmann *et al*., 1995; Qualmann *et al*., 1995; Dumoulin *et al*., 1998; Xiao *et. al*., 1999; Hansen *et.al.,* 2004; Lejeune *et al*., 2006; Blom *et al*., 2006; Salehi *et al*., 2008; Carr *et al*., 2008; Drewe *et al*., 2008; Kuhre *et al.,* 2015; ElHindawy *et.al*., 2017; Rachmiel et al., 2019).

However, the GI tract is a tube-like structure in chickens that develops and grows in length rapidly during the post-hatching period and then stops growing at a particular age. The only possibility then is for the mucosa to grow, which is primarily responsible for nutrient digestion and absorption. The presence of digested nutrients in the small intestinal lumen has been linked to anatomical and morphological changes in the mucosa (Yamauchi, 2002; Incharoen *et al.,* 2010). A study demonstrated that glucose and galactose could influence the development of enterocyte microvilli in mouse jejunum (Smith *et. al.,* 1991). On the other hand, many studies have shown that nutrients have an impact on the functional capacities of the intestinal mucosa in a variety of species, including chicken. As a consequence, it is feasible to change the morphological and functional characteristics of the intestinal mucosa by changing diet. As a result, the main objective of this dissertation was to uncover the underlying mechanisms of formation and function of the intestinal mucosa in the chicken influenced by dietary nutrients with the aid of various morphological, immunohistochemical and *in situ* hybridization approaches. There are three experiments that were carried out in accordance with the research procedure given in this dissertation, which is divided into the following chapters:

Chapter II

Dietary nutrient influences enterocyte microvilli

II. 1. Low dietary carbohydrate induces structural alterations in the enterocyte microvilli of chicken ileum

II. 1. 1. Introduction

Digestion and absorption of ingested nutrients occur in the small intestine, which mainly lines with absorptive epithelial cells known as enterocytes. These cells are concerned with moving nutrients from the intestinal lumen to circulating systems. Numerous tiny finger-like projections; microvilli, which uniformly cover its apical domain, therefore, characterize enterocytes. Microvilli contribute to increasing the surface area of enterocytes, thereby promoting the absorptive efficiency of luminal nutrients (Crawley *et al*., 2014; Helander and Fändriks, 2014). Morphological features of the intestinal epithelium also reflect the luminal state of the digestive organ because enterocyte microvilli have direct contacts with luminal contents.

On the basis of these facts, various kinds of cytostructural proteins influence maintaining of the shape and stability of the optimal functionality of microvilli. Among these proteins, actin is a major supportive type, which forms a core bundle consisting of 20~30 actin filaments with some actin-bundling proteins (Ohta *et al.,* 2012). Villin is one of main actin-bundling proteins in microvilli (Mooseker *et al.*, 1980; Coluccio and Bretscher, 1989). Several investigators also revealed that the structural characteristics of the microvilli were not static (Lecount and Grey, 1972; Craig and Powell, 1980; Stidwill *et al*., 1984). The structural features of microvilli have thus been intensely varied based on the state of actin and villin proteins. Several *in vitro* and *in vivo* studies found that alterations in actin microfilaments and villin levels modified the cytoskeleton of the microvillar core and finally altered the structural features of the microvilli (Stidwill and Burgess, 1986; Friederich *et al.,* 1989, 1999; Costa de Beauregard *et al.,* 1995; Revenu *et*

al., 2012;). Likewise, it has been demonstrated that adding and removing actin microfilaments in the cytoskeleton manipulated the microvillar shape (Mooseker and Tilney, 1975). However, another study indicated that the turnover rate of cytoskeletal core components in the microvilli was rapid *in vivo* (Stidwill *et al.*, 1984). Therefore, it was ascribed that the state of actin and villin proteins regulated the steady condition of the microvilli.

Glucagon-like peptide (GLP)-2 is a 33 amino acid peptide derived from proglucagon (PG) and is released from intestinal L cells in response to ingested nutrients (Drucker *et al.*, 1996; Xiao *et al.,* 1999). L cell in the chicken intestinal epithelium is an open type of endocrine cells with a long cytoplasmic process reaching the intestinal lumen (Nishimura *et al.*, 2013) and acts as a sensor to monitor ingested nutrients (Hiramatsu, 2020). Additionally, studies have shown that GLP-2 has many physiological actions on the gastrointestinal tract such as the promotion of mucosa and crypt cell proliferation and the suppression of apoptosis in epithelial cells. Hence, the maintenance of the integrity of epithelial cells for intestinal growth and absorption of nutrients is one of important actions of GLP-2 (Drucker *et al*., 1996; Drucker, 2001; Burrin *et al.,* 2003; Estall and Drucker, 2006).

For the last few decades, much interest has been shown in the structural arrangement of cytoskeletal core proteins that control the shape and stability of enterocyte microvilli. However, it is still unknown whether dietary carbohydrates (CHOs) influence the structural characteristics of enterocyte microvilli in both mammalian and avian species. In a recent study, I demonstrated that the dietary CHOs positively affected the proliferation of intestinal epithelial cells in the chicken ileum (Salahuddin *et al.*, 2021). Therefore, this research was primarily concerned with investigating the importance of

dietary CHOs in maintaining enterocyte microvilli by electron microscopy and immunohistochemistry in the chicken distal ileum. The effect of dietary CHO on the synthesis of GLP-2 in intestinal L cells was also clarified by using immunohistochemistry for GLP-2 peptide, *in situ* hybridization technique for proglucagon (PG) mRNA and morphometrical techniques.

II. 1. 2. Materials and Methods

The Committee for Animal Experiments reviewed the experimental protocol of this study, which was finally approved by the president of Shinshu University (Approval number: 300090).

1. 2. 1. *Animals and feeding management*

Twenty healthy male White Leghorn chickens were selected and divided into the control and three experimental groups (50%, 25%, and 0% CHO), each comprising five birds. The selection was based on their average body weight. Then, the control diet was provided to all chickens for three days to habituate them to the experimental diet. Following the habituation period, experimental feeding begun with each experimental diet being administered under controlled lighting (12 hr light: 12 hr darkness) for seven days. Additionally, chickens had access to feed and water *ad libitum*. Feed intake and body weight of each chicken were also measured daily at the same clock time during the experimental period.

The composition of the control and experimental diets was shown in Table 1. As shown, the CHO contents in the experimental diets for 50%, 25%, and 0% groups were 50%, 25%, and 0% of that in the control diet, respectively. The source of CHO (cornstarch) in the experimental diets was also the same as that in the control group.

Furthermore, the metabolizable energy (ME) in each group was adjusted to a similar level among the four groups by adding corn oil. Thus, the ME level (2,850 kcal/kg) of the diet was similar to that of the ME requirement, which was recommended by the Japanese Feeding Standard for Poultry (NARO, 2011).

1. 2. 2. *Tissue samples*

Chickens were sacrificed by decapitation under the anesthetic condition by an intravenous injection of sodium pentobarbital (64.8 mg/kg body weight). The distal part of the ileum was then dissected out from each chicken as a tissue sample, after which its luminal contents were washed out with a 0.75% NaCl solution. For light microscopy, tissue samples were immersed in Bouin's solution at room temperature for 24 hr and embedded in paraffin wax in the ordinary manner. For electron microscopy, a part of tissue samples of three birds randomly selected from each group was cut into small pieces with a razor blade and immersed in Karnovsky's fixative (a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 50 mM cacodylate buffer) at 4℃ for 2 hr. After washing with 50 mM cacodylate buffer, pieces of tissue samples were postfixed with 1% osmium tetroxide (OsO_4) in 0.2 M phosphate buffer at 4℃ for 2 hr, then embedded in epoxy resin (Quetol 812, Nisshin EM, Tokyo, Japan) in an ordinary manner.

1. 2. 3. *Electron microscopy*

Ultra-thin sections were cut from each epoxy block using an ultramicrotome (SuperNova, Reichert-Jung, Vienna, Austria) and treated with 10% (v/v) TI Blue (Nisshin EM, Tokyo, Japan) and 2% (w/v) lead citrate for electron staining. Subsequently, they were observed and photographed under a transmission electron microscope (JEM-1400; JEOL, Tokyo, Japan). According to the previously described method (Brown, 1962), the

length, width and linear density of enterocyte microvillus were measured by using ImageJ software (Fiji, National Institutes of Health, Bethesda, MD, USA). Width of the microvillus was measured at its bottom position. Three hundred microvilli were randomly selected from plural enterocytes of each chicken. A total of nine hundred microvilli was measured from three chickens of each group for the measurement of length and width. Linear density means the number of microvilli present per unit of length on the apical cell surface. To determine the linear density of microvilli, forty areas were randomly selected from three chickens and a total of one hundred and twenty areas were measured in each group. The linear density of enterocyte microvilli was described as the number of microvilli per μm in length of the apical cell surface.

1. 2. 4. *Immunofluorescence technique for villin and β-actin*

Immunofluorescence technique was conducted to detect the immunoreactivity of β-actin and villin in the distal ileum. Paraffin sections treated with 10% normal goat serum were firstly incubated with mouse anti-villin monoclonal antibody (sc-58897, diluted to 1:20, Santa Cruz Biotechnology, Dallas, TX, USA) or rabbit antiserum against β-actin (GTX109639, 1:5000, Gene Tex, Irvine, CA, USA) for 24 hr. After several washing with phosphate buffered saline (PBS), sections were incubated with DyLight 488-labeled goat anti-mouse IgG antibody (610-141-121, 1:300, Rockland, PA, USA) or DyLight 549-labeled goat anti-rabbit IgG antibody (611-142-122, 1:300, Rockland, PA, USA) for 3 hr. Subsequently, these sections were coverslipped with an aqueous mounting medium (Perma Fluor; Thermo Fisher Scientific, Fremont, CA, USA), observed and photographed under a fluorescence microscope (AxioImagerA1; Zeiss, Göttingen, Germany). All incubations were carried out in a moisture chamber at room temperature. Specificity of primary antibodies used in this study were examined by the manufacturers.

1. 2. 5. *Immunohistochemistry for GLP-2*

The streptavidin-biotin technique was used to identify GLP-2-immunoreactive cells in the distal ileum according to the previously applied protocol (Hiramatsu and Ohshima, 1995). Paraffin sections were treated with an antigen retrieval agent (ImmunoSaver, Nisshin EM, Tokyo, Japan) at 98℃ for 45 minutes. They were incubated with rabbit anti-human (Arg^{34})-GLP-2 serum (1:2,000, H-028-14, Phenix Pharmaceuticals, Burlingame, CA, USA) as a primary antibody for 24 hr following the treatment with 10% normal goat serum (50062Z, Invitrogen, Carlsbad, CA, USA) for 20 minutes. After several washing with PBS, sections were incubated with biotin-labeled goat anti-rabbit IgG serum (1:300, AP132B, Millipore, CA, USA) followed by poly-HRP20-labeled streptavidin (1:300, SP20C, Stereospecific Detection Technologies, Baesweiler, Germany). The immunocomplex was visualized with 0.05% 3,3-diaminobenzidine solution in tris-HCl buffer. Sections were coversliped after counterstaining with Mayer's hematoxylin, observed and photographed under a light microscope. The specificity of the primary antibody used in this study was tested in our previous study (Nishimura *et al*., 2013). All incubations were carried out in a moisture chamber at room temperature.

Morphometrical evaluation of GLP-2-immunoreactive cells was conducted using a computerized image analyzing system (KS400, Carl Zeiss, Gottingen, Germany) according to our previous method (Hiramatsu *et al*., 2005). To assess the frequency of occurrence, the number of GLP-2-immunoreactive cells with clearly visible nuclei was counted. Subsequently, the mucosal layer area was measured to calculate the cell number per mucosal area (cells/mm^2). Then, twenty areas were randomly selected from each bird, and one hundred areas in total were measured from five birds in each group for the morphometry.

1. 2. 6. *In situ hybridization for proglucagon (PG) mRNA*

In situ hybridization technique was conducted to identify cells expressing PG mRNA signal according to the previously described technique (Watanabe *et al*., 2014). A commercially available kit (IsHyb In Situ Hybridization Kit; Biochain Institute, Newark, CA, USA) was used to hybridize and visualize digoxigenin (DIG)-labeled probes according to the manufacturer's instructions. Sections were incubated with an anti-DIG antibody diluted in alkaline-phosphatase solution (1:500), and a mixture solution of nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl-phosphate was applied to visualize the conjugated probe under a light microscope. All oligonucleotide probes were designed according to the PG mRNA sequence (Richards and McMurtry, 2008). These probes were commercially synthesized (BEX, Tokyo, Japan) in this study. The sequences of the PG mRNA antisense and sense probes are given Table 4. Notably, a sense probe was applied as a negative control for PG mRNA.

Cells expressing PG mRNA signal were counted using a computerized image analyzing system as mentioned above and the frequency of occurrence of these cells per mucosal area (cells/mm^2) was obtained. Twenty areas were randomly selected from the preparations of each bird. A total of one hundred areas from five chickens of each group were examined to calculate the frequency of occurrence of cells expressing PG mRNA signal.

1. 2. 7. *Statistical analysis*

Statistical analysis for all data was conducted using SAS (SAS Inst. Inc., Cary, NC, USA). The GLM was performed in one way analysis of variance manner. Tukey`s test

was applied to discover the differences in mean values among all groups. The $P<0.05$ was set as a statistically significant standard.

II. 1. 3. Results

There were no significant differences in the daily feed intake and body weight gain of chickens during the experimental period among four groups. However, both values decreased in the experimental groups compared with those in the control group.

1. 3. 1. *Electron microscopy of enterocyte microvilli*

Electron micrographs of enterocyte microvilli in the distal ileum of the control and three experimental groups are shown in Fig. 1. There were obvious differences in the appearance of microvilli between the control group and lower CHO level, 25% and 0%, groups. Straight and tightly packed microvilli showing conditions were observed in the control and 50% groups (Fig. 1). In contrast, the fragmentation of microvilli (Fig. 1, black arrows in top panels) was dominantly observed in the lower CHO level groups, especially in the 0% group. Wide gap spaces were also observed between microvilli in 25% and 0% groups (Fig. 1, white arrows in bottom panels). Many small vacuoles were contained in enterocytes with the fragmentation of microvilli of 25% and 0% groups (Fig. 1, asterisks in top panels). The limited membrane of these vacuoles was electron-dense and thick.

Morphometrical data obtained by electron microscopy are shown in Fig. 2. The length of enterocyte microvilli was significantly longer in the control group than in the experimental groups ($P<0.05$), which showed shorter microvilli as the dietary CHO levels decreased. Microvilli in 0% group were significantly shorter than those in 50% and 25% groups ($P<0.05$) (Fig. 2A). However, no significant differences were observed in the length of microvilli between 50% and 25% CHO groups. The width of enterocyte

microvilli was also significantly wider in the control than the experimental groups (P<0.05), which showed narrower microvilli as the dietary CHO level decreased. Significant differences were observed in the width of microvilli between 50% and the lower CHO level groups (P<0.05). There were no significant differences in the width of microvilli between 25% and 0% groups (P<0.05) (Fig. 2B). Significant differences in the linear density of enterocyte microvilli were observed among the four groups (P<0.05) (Fig. 2C). The linear density of microvilli was significantly higher in the control than the experimental groups and decreased as the dietary CHO level decreased (P<0.05) (Fig. 2C). 0% group showed the lowest value of linear density of microvilli among the four groups.

1. 3. 2. *Immunoreactivity for villin and β-actin*

Immunoreactivities for villin and β-actin were observed in the distal ileum of all groups (Fig. 3). The intensity of immunofluorescence for villin was comparatively weaker in groups administered lower CHO levels (25% and 0%) than the control group (Fig. 3, top panels). No obvious differences in the staining intensity of villin were observed between 25% and 0% groups. The intensity of immunofluorescence for β-actin was also weaker in 0% group than the other groups (Fig. 3, bottom panels). The staining intensity of β-actin in 25% group was apparently higher than that in 0% group (Fig. 3, bottom panels).

Additionally, no differences were found in the distributional patterns of villin- and β-actin-immunoreactive sites among the four groups. Immunoreactivities for villin and β-actin were localized in the brush border region of the villus epithelium. Likewise, no immunoreactivities for villin and β-actin were observed in crypts of all groups.

1. 3. 3. *GLP-2-immunoreactive cells*

Many endocrine cells immunoreactive for GLP-2 antiserum were found in the epithelium of the distal ileum from all groups. Most GLP-2-immunoreactive cells were scattered in the epithelium from the middle part of the villi to the crypts (Fig. 4A-a and -d). GLP-2-immunoreactive cells in the control group showed pyramidal- or spindle-like shape with a long cytoplasmic process in the villus epithelium (Fig. 4A-b, arrow) and comma-like shape in the crypts (Fig. 4A-c). In contrast, GLP-2-immunoreactive cells having oval or round shapes were frequently observed in the lower CHO level groups, especially in the 0% group (Fig. 4A-e and f, arrows). There were no obvious differences in the distributional pattern of GLP-2-immunoreactive cells among the four groups.

There were significant differences in the frequency of occurrence of GLP-2-immunoreactive cells among four groups ($P<0.05$). The control group showed a significantly higher frequency of GLP-2-immunoreactive cells compared to those in the experimental groups ($P<0.05$). The frequency of occurrence of GLP-2-immunoreactive cells decreased with declining the dietary CHO level and showed the lowest value in 0% group (Fig. 4B).

1. 3. 4. *Cells expressing proglucagon (PG) mRNA signals*

Cells expressing PG mRNA signals were observed in the distal ileum of the control and three experimental groups (Fig. 4C). Cells expressing PG mRNA signals were scattered in the epithelium of the bottom part of the villi and crypts. No obvious differences were observed in the distributional pattern of these cells among the four groups.

There were significant differences in the frequency of occurrence of cells expressing PG mRNA signal ($P<0.05$) (Fig. 4D) among four groups. 25% and 0% groups showed extremely lower values of the frequency of occurrence of cells expressing PG mRNA than the control and 50% groups.

II. 1. 4. Discussion

This study demonstrated that dietary CHOs contributed to the maintenance of enterocyte microvilli in the chicken ileum and suggested that the induction of GLP-2 secretion from intestinal L cells could be concerned in this maintenance system.

Enterocyte microvillus is the main structure at the apical domain of enterocytes and increases the free cell surface area for the efficient absorption of ingested nutrients (Helander and Fändriks, 2014). This morphological adaptation allows the intestinal epithelium to make close and prolonged contact with luminal nutrients (Crawley *et al*., 2014). Hence, ingested nutrients can affect the structure of enterocyte (Smith, 1992). My previous study demonstrated that dietary CHOs positively affected the proliferation of intestinal epithelial cells including enterocytes and goblet cells in the chicken ileum (Salahuddin *et al*., 2021). In this study, microvilli showed ultrastructural alterations such as fragmentation and wide gaps in microvilli obtained from 25% and 0% groups. These findings provide evidence that microvilli fall down from the apical surface of enterocyte under CHO-free condition. Morphometrical data obtained from electron microscopy also demonstrated structural changes of microvilli such as shorter length, narrower width and lower density in 25% and 0% groups than in the control group. These morphological data therefore indicate that the dietary CHO would be the essential nutrient to maintain the structure of enterocyte microvilli. Additionally, many vacuoles with electron-dense and thick limiting membrane were observed in enterocytes showing the fragmentation of

microvilli from 25% and 0% groups. The appearance of these vacuoles may indicate the disturbance of enterocyte induced by CHO-free condition. However, more systematic analysis is necessary to this phenomenon.

Microvillus contains a conspicuous cytoskeletal core consisting of about 20 to 30 actin filaments that are bundled by actin bundling proteins, villin, ezrin and fimbrin (Bretscher and Weber, 1980; Mooseker *et al*., 1980; Fath and Burgress, 1995). Villin is a major and versatile actin regulatory protein that regulates biological functions in enterocytes, such as cell morphology and actin dynamics (Khurana and George, 2008). This actin binding protein is also proposed to participate in the organization and stabilization of the brush border core bundle (Friedrich *et al*., 1999). Dreckhahn and Dermietzel (1988) revealed that villin was found within the entire microvillus filament bundles in chicken intestinal epithelium. Inhibition of specific protein synthesis was also related to the cytoskeletal core exhibited disruption of the microvillar structure in the small intestine of chickens (Lecount and Grey, 1972). Moreover, cytoskeletal core proteins showed a quick turnover, thereby accounting for the stability of microvillar shapes in mature epithelial cells of chicken (Stidwill *et al.*, 1984). In the present study, I demonstrated that immunoreactivities for β-actin and villin were localized along with the epithelial surface of the chicken ileum in the control group. However, intensities of immunofluorescence for these structural proteins were very weak in 0% group compared to the control group. Revenu *et al*. (2012) showed the perturbation of microvilli in mouse model lacking actin binding proteins including villin, but this alteration was minimal in a mouse model lacking villin only. Similarly, this study revealed obviously lower immunoreactivity for β-actin on the epithelial surface of the ileum obtained from 0% group. This finding therefore indicated the ravel of actin filament bundles in microvilli. On the basis of these

results, we propose that the CHO-free diet induced the reduction of villin and other actin binding proteins synthesis followed by the desquamation of microvilli, which was observed by electron microscopy as mentioned above.

GLP-2 is a peptide derived from PG and secreted from intestinal L cells which has a long cytoplasmic process in contact with the intestinal lumen. This peptide shows many important actions, such as the stimulation of intestinal growth, the increase of crypt cell proliferation and the reduction of epithelial apoptosis (Drucker *et al*., 1996; Tsai *et al.,* 1997; Litvak *et al.*, 1998; Burrin *et al.*, 2007). In the chicken small intestine, GLP-2-immunoreactive cells were mainly localized in the lower part of villi and crypts of the distal ileum (Monir *et al.*, 2013). GLP-2 was colocalized with GLP-1 in the same secretory granule of L cells which showed the open-typed form of endocrine cells (Nishimura *et al*., 2013). EECs in the open-typed form are considered as the primary chemosensory agent to sense nutrients in the intestinal lumen (Breer e*t al.*, 2012) and secret hormones in response to ingested nutrients (Gribble and Reimann, 2016). Some studies found that the ingestion of CHOs increased the secretion of GLP-2 in the mammalian small intestine (Xiao *et al*., 1999; Rachmiel *et al.*, 2019). Noticeably, the frequency of occurrence of GLP-2-immunoreactive cells, in this study, was significantly higher in the control group than in the experimental groups. A declining tendency of the frequency of these cells was also observed with decreasing dietary CHO levels. Moreover, the lower CHO level groups, especially 0% group, showed many GLP-2-immunoreactive cells in round or oval shape. Such cells were frequently found in the ileal epithelium obtained from fasted chickens and contained vacuoles in the perikaryon having small lobule nuclei (our unpublished data). Similarly, results from this study indicated that the GLP-2-immunoreactive cells found in the lower dietary CHO level groups would be

abolished from the epithelium due to lack of proper quantities of CHO in the lumen. I propose low CHO levels as the main reason for the reduced frequency of occurrence of the GLP-2-immunoreactive cells in lower CHO level groups, especially in 0% group.

In situ hybridization also demonstrated that frequencies of occurrence of cells expressing PG mRNA signal in 25% and 0% groups were significantly decreased compared with that of the control group. This finding indicates the possibility that dietary CHO influences the transcription level of PG mRNA in L cells of the chicken ileum. Previous results from two research groups showed that the transcription level of PG mRNA in L cell line was upregulated as treated with higher level glucose (Daoudi *et al.*, 2011; Puddu *et al.*, 2014). Their studies strongly support this possibility. Therefore, it is possible that low dietary CHO levels can impair PG mRNA transcription and reduce the frequency of GLP-2-containing cells in chicken distal ileum.

Another study showed that multiple actions of GLP-2 on intestinal epithelium were not directly targeted for crypt or epithelial cells, because GLP-2 receptor (GLP-2R) was not found on these cells (Dube *et al*., 2006). GLP-2 activates its specific GLP-2R in the intestinal subepithelial myofibroblast cells that release insulin-like growth factor (IGF)-1. Released IGF-1 couples with IGF-1 receptor (IGF-1R) in crypt cells and stimulates the proliferation of crypt cells. Therefore, it is proposed that GLP-2 released in response to CHO ingestion stimulates the proliferation of enterocytes via IGF-1 system in the chicken small intestine. Bulut *et al*. (2004, 2008) demonstrated that GLP-2 effects on intestinal epithelial cells repair are transforming growth factor-β dependent. Similarly, Ørskov *et al.* (2005) revealed that keratinocyte growth factor might be responsible for the intestinal proliferation induced by GLP-2. These growth factors from the intestinal subepithelial

myofibroblast cells were also proposed to be concerned with this mechanism. However, more systematic studies are necessary to clarify this molecular mechanism.

In this study, corn oil as a fat source was added at a higher inclusion level for maintaining the same energy level in the diets of the control and three treatment groups. High fat levels had no significant effects on results, because dose-dependent changes induced by corn oil were not found in any experiments conducted in this study.

II. 1. 5. Conclusion

These results demonstrate that dietary CHO is the most important nutrients that maintain the enterocyte microvilli in chicken ileum. This maintenance system depends on GLP-2 secretion and the stimulation of PG mRNA transcript level in the intestinal L cells.

Table 1

Composition of the experimental diets

Composition (g)	Control	50%	25%	0%
Isolated soyabean protein	217.4	217.4	217.4	217.4
L-Cysteine	0.7	0.7	0.7	0.7
L-Methionine	0.9	0.9	0.9	0.9
L-Threonine	0.4	0.4	0.4	0.4
Cornstarch	491.4	245.7	122.9	0.0
Cellulose	194.7	340.2	412.9	485.7
Corn oil	30	130.2	180.3	230.4
Mineral mixture	60.0	60.0	60.0	60.0
Vitamin mixture	2.0	2.0	2.0	2.0
Choline chloride	1.5	1.5	1.5	1.5
Inositol	1.0	1.0	1.0	1.0
Total (g)	1000.0	1000.0	1000.0	1000.0
Me (Kcal/kg)	2850	2850	2850	2850
CP%	18	18	18	18

Composition of the diets was calculated according to NRC (1994)

Isolated soybean protein (CP=82.8%)

CP : Crude Protein

ME : Metabolizable energy.

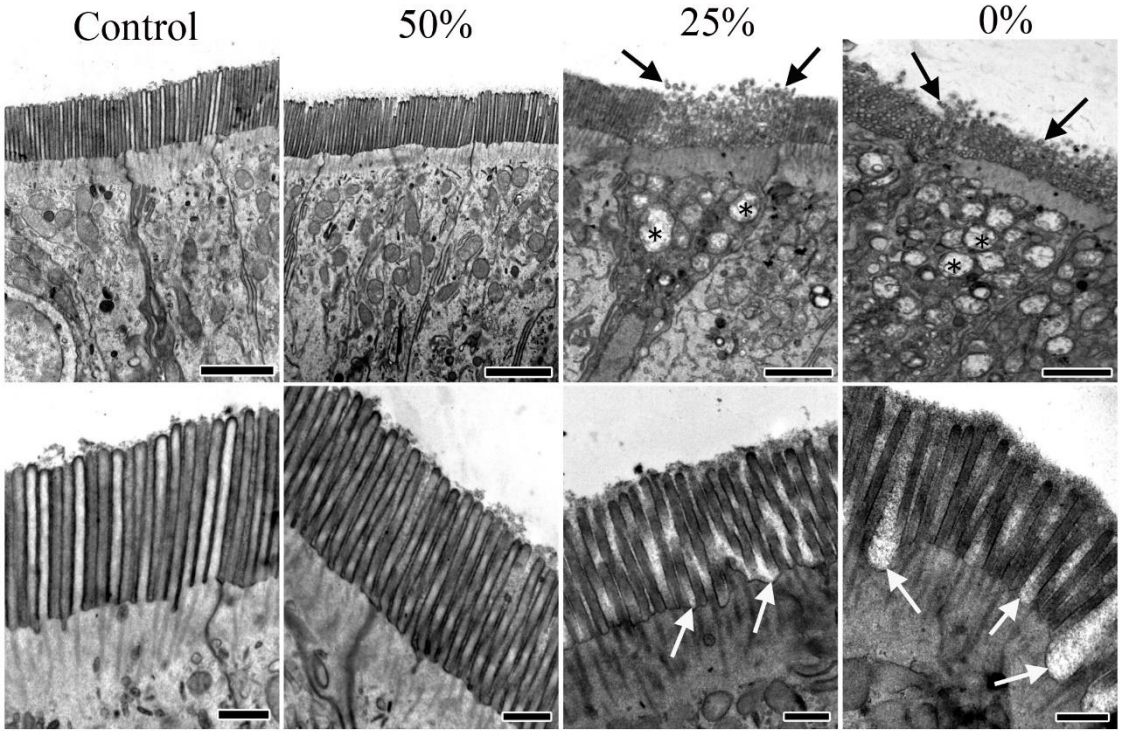

Fig. 1. Electron microscopy of the apical surface of enterocytes from the chicken ileum of the control, 50%, 25% and 0% groups. Black arrows and asterisks in the top panel of 25% and 0% groups indicate the fragmentation of microvilli and vacuoles, respectively. White arrows in the bottom panel of 25% and 0% groups indicate gap spaces between neighbor microvilli. Scale bars in the top panel: 2 μm. Scale bars in the bottom panel: 1 μm**.**

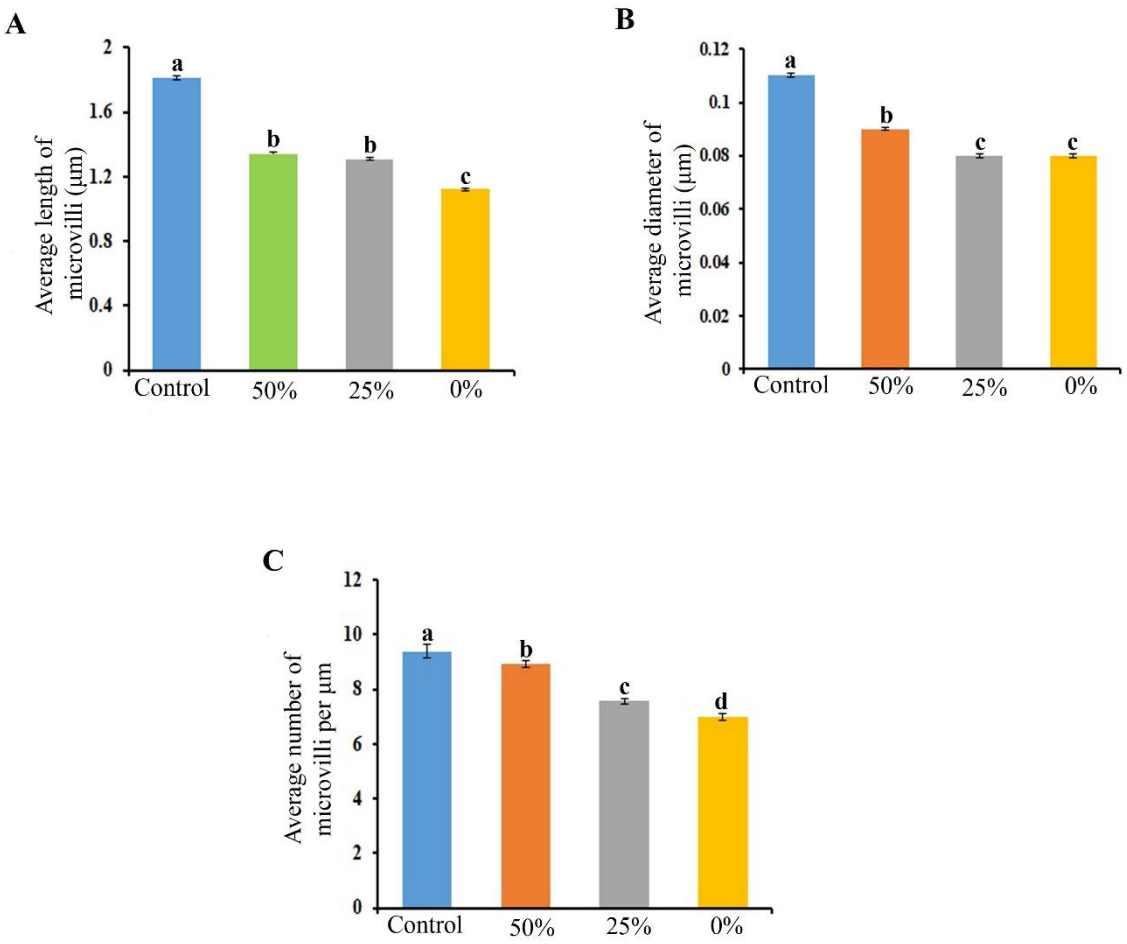

Fig. 2. Morphometrical parameters of enterocyte microvilli from the chicken ileum of the control, 50%, 25% and 0% groups. (**A**) Average length of microvilli (µm). (**B**) Average width of microvilli (µm). (**C**) Average linear density of microvilli (microvillus/µm apical surface of enterocyte). All parameters are significantly lower in 0% group than the control group. There is a significant deference between different alphabets in each figure at $P<0.05$. a>b>c>d.

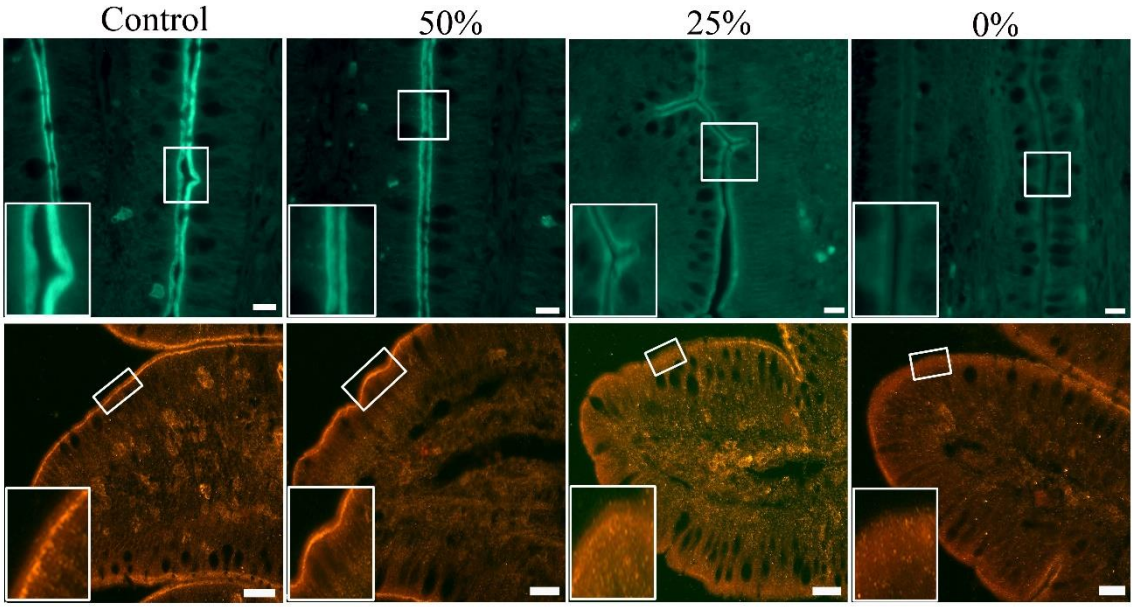

Fig. 3. Villin (top panels) and β-actin (bottom panels) immunoreactivities in chicken ileum of the control, 50%, 25% and 0% groups. Villin immunofluorescence is observed at the apical site of epithelial cells in the control and three experimental groups, but is obviously weaker in 0% group than the control group. β-actin immunofluorescence is also observed at the apical site of epithelial cells in the control and three experimental groups, but is obviously weaker in 0% group than the control group. Scale bar= 20μm.

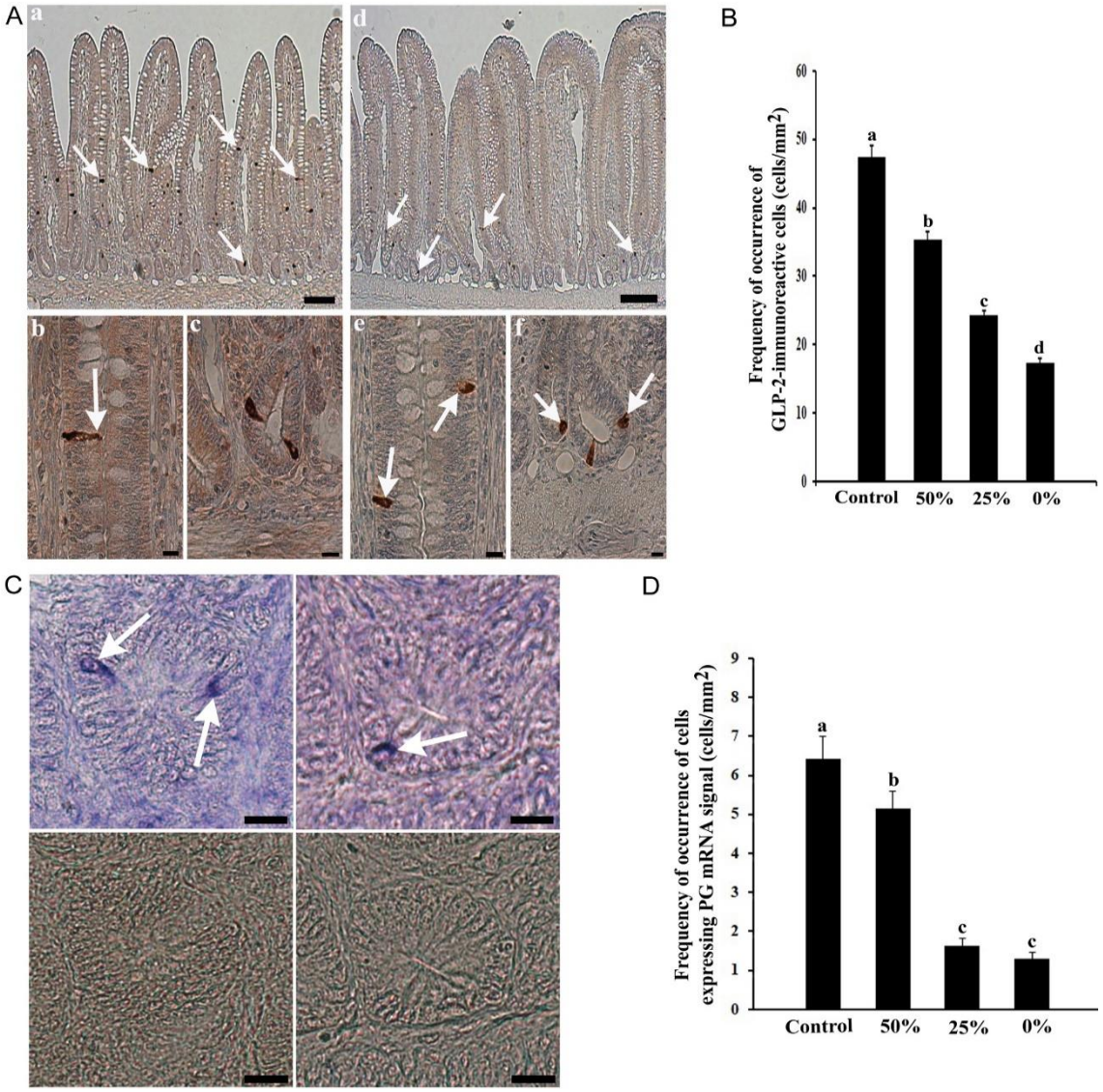

Fig. 4. Glucagon-like peptide (GLP)-2-immunoreactive and PG mRNA-expressing cells in chicken ileum of the control and 0% groups. (A) Photomicrographs showing the distributional pattern of GLP-2 immunoreactive cells (arrows) in chicken ileum of the control (a) and 0% (d) groups. GLP-2-immunoreactive cells in villus epithelium (b) and crypt (c) from the control group have a cytoplasmic process in contact with the intestinal lumen, but those cells from 0% group have an oval or round shape in both villus epithelium (e) and crypt (f). (B) Density of occurrence of GLP-2 immunoreactive cells in ileum of the control and three experimental groups. There is a significant difference between different alphabets. $P<0.05$. (C) Proglucagon (PG) mRNA-expressing cells (arrows) in chicken ileum from the control (left panels) and 0% (right panels) groups. Negative control sections using sense probe shows no cells expressing PG mRNA (bottom panels). (D) Frequency of the occurrence of PG mRNA-expressing cells in the chicken ileum from the control and three experimental groups. There is a significant difference between different alphabets. $P<0.05$.

II. 2. Summary

This study was taken to evaluate the effect of dietary CHO on the enterocyte microvilli in the distal ileum of chicken. Male White Leghorn chickens were divided into three experimental groups and a control group. The experimental groups' diets included 50%, 25%, and 0% of the carbohydrate level of the control diet. I evaluated the structural changes in ileal enterocytes using a transmission electron microscope and an immunofluorescent method for β-actin and villin. Additionally, immunohistochemistry and *in situ* hybridization were used to identify glucagon-like peptide (GLP)-2 and proglucagon mRNA, respectively. The higher CHO groups had straight and densely packed microvilli. On the other hand, microvilli were disorganized and fragmented in the lower CHO groups. Additionally, the electron microscopic parameters were decreased when the level of CHO was decreased in the diet. The 0% group had substantially lower immunofluorescence intensities for β-actin and villin than the control group. Similarly, the frequency of GLP-2-immunoreactive and proglucagon mRNA-expressing cells decreased when dietary carbohydrate levels decreased. The present findings indicate that dietary CHO is a potential nutrient that might alter mucosal development in the chicken distal ileum. It is expected that dietary CHO would increase the mucosal surface area, which will facilitate in the digestion and absorption of nutrients. These findings may therefore be utilized as an indicator by poultry nutritionists when formulating feed and conducting further nutritional study.

Chapter III

Dietary nutrient influences endocrine cells

III. 1. Dietary protein level influences on neurotensin-immunoreactive cells in the chicken ileum

III. 1. 1. Introduction

Gastrointestinal tract is the main site involved in the process of digestion and absorption of ingested nutrients and also contains numerous endocrine cells which secret many kinds of peptide hormones including neurotensin. Neurotensin is a tridecapeptide separated from the extracts of bovine hypothalamus (Carraway and Leeman, 1973). The precursor protein of this hormone is consisted of 169-170 amino acids and exceptionally conserved polypeptide in rat, dog, and cow (Dobner *et al*., 1987; Kislauskis *et al*., 1988). Neurotensin has been found in the central and peripheral nervous systems as well as in the gastrointestinal tract of various animal species (Polak and Bloom, 1982; Reinecke, 1985) and exerts enormous physiological functions in the gastrointestinal tract such as the regulation of gastric and intestinal motility (Thor and Rosell, 1986), the induction of pancreatic and biliary secretion (Gui *et al*., 2001), the enhancement of mucosal growth (Evers, 2006), inflammatory response (Gross and Pothoulakis, 2007), and the increase of capillary permeability (Harper *et al*., 1984).

EECs are designated as letter(s) and neurotensin-secreting cell is named as N cell. Helmstaeder *et al*. (1977) and Sundler *et al*. (1982) found that N cell in the mammalian intestine had an apical surface covered with microvilli and stored secretory granules in its basal cytoplasm. These morphological data indicate that intestinal N cell is the endocrine cell of open-type and may respond to the intraluminal nutrients. Several studies carried out in vitro and in vivo showed that nutrients such as amino acids, glucose and lipid stimulated neurotensin secretion from the intestine of mammalian species (Rosell and Rökaeus, 1979; Go and Demol, 1981; Holst Pedersen *et al*., 1988; Dumoulin *et al*., 1998;

Drewe *et al*., 2008; Kuhre *et al*., 2015). Atoji *et al*. (1994) revealed that neurotensin-immunoreactive cells of open type were distributed in the whole gastrointestinal tract of several avian species. DeGolier *et al*. (2013) demonstrated that neurotensin was released from the chicken duodenum in response to intraluminal contents. Glucagon-like peptide (GLP)-1 is one of major peptide hormones secreted from the chicken intestine. Recent studies in chickens demonstrated that GLP-1 secretion was influenced by ingested protein (Monir *et al*., 2014a) and that GLP-1 colocalized with neurotensin in the same endocrine cells of ileum at a high ratio (Nishimura *et al*., 2017). These data suggest that neurotensin is also released from chicken ileum in response to the ingested protein. However, it is not clear if dietary protein was a trigger of neurotensin secretion in the chicken small intestine, as we know. In the present study, I aimed to clarify the influence of dietary crude protein (CP) levels on neurotensin-containing endocrine cells in the chicken ileum by immunohistochemical and morphometrical techniques.

III. 1. 2. Materials and Methods

1. 2. 1. *Experimental birds and feeding*

Twenty white leghorn chickens at 6 weeks of age were used in this study. They were divided into four groups, CP18% (control), CP9%, CP4.5% and CP0%, with 5 heads each and fed with the experimental diets containing different CP levels, 18%, 9%, 4.5% and 0%, for 7 days after feeding with the control diet for 3 days. Details of dietary composition used and experimental feeding procedures in the present study are described in Table 2.

1. 2. 2. *Tissue samples*

After completing the experimental protocols, chickens were sacrificed by decapitation under anesthetic condition. The distal ileum about 2 cm in length was

immediately dissected out from each chicken as the tissue sample. Neurotensin is colocalized with GLP-1in the same cell at a high ratio in the distal ileum (Nishimura *et al.*, 2017). After washing with 0.75% sodium chloride solution, tissue samples were fixed in Bouin's solution for 24 hr at room temperature and embedded in paraffin wax in the ordinary manner. Paraffin sections were cut at 5 μm with sliding microtome and supplied to immunohistochemistry for the detection of neurotensin.

1. 2. 3. *Immunohistochemistry*

The streptavidin-biotin method was used for the detection of neurotensin-immunoreactive cells (Guesdon *et al*., 1979). Paraffin sections treated with normal goat serum were incubated with rabbit polyclonal antibody against neurotensin (AB5496, EMD Millipore, CA, USA, diluted to 1:2000) at room temperature for 24 hr. Biotin-labeled goat anti-rabbit IgG (AP132B, Millipore, CA, USA, diluted to 1:100) and streptavidin-polyHRP20 (SP20C, Stereospecific Detection Technologies, Baesweiler, Germany, diluted to 1:300) were used as the secondary antibody and the label for visualization of immunocomplex, respectively.

1. 2. 4. *Morphometry*

Morphometrical analysis was performed as described in our previous study (Hiramatsu *et al*., 2005). Cells showing neurotensin-immunoreactivity were counted in each group with only clearly identifiable nuclei and the area of the mucosal layer was measured. The cell number per area of the mucosal layer (cells/mm^2) was then calculated as the frequency of occurrence of neurotensin-immunoreactive cells. These measurements and quantifications were carried out using a computerized image analyzing

system (KS400; Zeiss, Göttingen, Germany). Twenty areas were measured in each bird. One hundred areas in total were measured from five chickens in each group

1. 2. 5. *Statistical analysis*

Statistical analysis was conducted to determine the differences in the frequency of neurotensin-immunoreactive cells among the four groups using Tukey's method (Yanai, 2011).The GLM procedure (SAS/STAT) was carried out for multiple regression analysis between the daily protein intake and the frequency of occurrence of neurotensin-immunoreactive cells. The average value of the daily protein intake (g/day) of each chicken during the experimental period was calculated from the average value of the daily feed intake and CP level (%) of the experimental diet. Based on the national and the institutional regulations and guidelines, all procedures of animal experiments were reviewed by the Committee for Animal Experiments of and finally approved by the president of Shinshu University.

III. 1. 3. Results

Many endocrine cells showing immunoreactivity for neurotensin antiserum were found in the ileal epithelium of all groups. Neurotensin-immunoreactive cells were mainly found in the epithelial of the intestinal villi and crypts in all the groups (Fig. 5a-d). There was no obvious difference in the distributional pattern of these cells among four groups. Neurotensin-immunoreactive cells had flask- or spindle-like shape in the villus epithelium (Fig. 6a, c) and comma-like shape in the crypt (Fig. 6b, d), with apical cytoplasmic process reaching to the intestinal lumen. However, neurotensin-immunoreactive cells in round or oval shape were observed in the lower dietary CP level groups, especially in CP0% group (Fig. 6c, arrow). Frequency of occurrence of

neurotensin-immunoreactive cells in CP18%, CP 9%, CP 4.5% and CP 0% groups were 42.4 ±3.3, 36.6 ±2.2, 30.8 ±2.6 and 25.4 ±3.8, respectively (Fig. 7, cell number per mucosal area: cells/mm^2, mean ± SD). Significant differences were observed in the frequency of occurrence of neurotensin-immunoreactive cells between the control and lower CP level, CP 4.5% and 0%, groups (P<0.05). The values of frequencies were found a decreasing fashion with the reducing level of dietary protein. There was a significant strong positive correlation between the daily protein intake (X) and the frequency of occurrence of neurotensin-immunoreactive cells (Y) measured by using the GLM procedures for multiple regression analysis. The regression equation was: Y = 25.92 + 1.87 X (Fig. 8).

III. 1. 4. Discussion

The present study demonstrated that the dietary protein level influenced neurotensin secretion from intestinal N cells of the chicken ileum.

Immunohistochemistry in this study showed that EECs exhibiting neurotensin immunoreactivity were scattered in the villus and crypt epithelium of the chicken ileum from the control and three experimental groups. These neurotensin-immunoreactive cells have a long cytoplasmic process in contact with the intestinal lumen as mentioned in Atoji *et al*. (1994). This type of EECs is classified into open-type (Hiramatsu, 2020). Open-type enteroendocrine cell receives intraluminal nutrients as a signal and releases its hormone into blood circulation (endocrine) or extracellular space nearby (paracrine) (Gribble and Reimann, 2016). Immunohistochemical and ultrastructural studies in mammals indicated that intestinal N cells of open-type had an apical surface covered with microvilli and contained secretory granules in its basal cytoplasm (Helmstaeder *et al*., 1977; Sundler *et al*., 1982). Microvilli of open-type endocrine cells supply a receptor site

for chemical signals, such as nutrients, in the intestinal lumen (Hashimoto *et al*., 1999; Breer *et al*., 2012). Sundler *et al*. (1982) reported neurotensin-immunoreactive cells having an apical process furnished with microvilli in the chicken antrum. A previous study demonstrated the similar ultrastructural feature of chicken intestinal L cells which secret glucagon-like peptide (GLP)-1 (Nishimura *et al*., 2013) and that a large part of GLP-1-immunoreactive cells also exhibited immunoreactivity for neurotensin in the chicken ileum (Nishimura *et al*., 2017). It is possible that neurotensin-immunoreactive cells observed in this study have an apical process covered with microvilli and receive nutrients as the chemical signal.

Several *in vivo* and *in vitro* studies reported stimulative effects of luminal nutrients, especially fats and fatty acids, on the secretion of neurotensin in mammals (Rosell and Rökaeus, 1979; Go and Demol, 1981; Holst Pedersen *et al*., 1988; Dumoulin *et al*., 1998; Drewe *et al*., 2008; Kuhre *et al*., 2015) and chicken (DeGolier *et al*., 2013). *In vitro* study of Dumoulin *et al*. (1998) showed that infusion of peptone, an enzymatic digest of protein, induced a marked increase of neurotensin from the rat ileum. However, it is not clear if ingested protein has influences on neurotensin secretion directly. Morphometrical analysis in this study revealed that there were significant differences in the frequency of occurrence of neurotensin-immunoreactive cells between the control and lower CP level, 4.5% and 0%, groups. The frequency of neurotensin-immunoreactive cells was highest in the control group and was reduced with the decreasing of the dietary CP level. Moreover, multiple regression analysis showed a strongly positive correlation between frequencies of occurrences of neurotensin-immunoreactive cells and daily protein intake. El-Salhy *et al*. (2016) described that a change in diet not only affected the release of gastrointestinal hormones, but also altered the densities of the gut endocrine cells. In fact, the change of

the dietary protein level altered the frequencies of occurrence of GLP-1-immunoreactive cells in the chicken ileum (Monir *et al*., 2014a). In addition, neurotensin-immunoreactive cells in round or oval shape were found in lower CP level groups, especially in CP 0% group. Endocrine cells in a round or oval shape were found in the ileal epithelium of the fasted chicken and degenerating features, i.e.; vacuoles in the perikaryon and lobulated nuclei, were observed in these cells at the ultrastructural level (our unpublished data). This phenomenon suggests that neurotensin-immunoreactive cells are going to be degenerated in the CP 0% group because of the absence of luminal proteins. Thus, the present morphometrical data indicate that the dietary protein is one of important factors which control activities of N cells in the chicken small intestine.

Colocalization of plural hormones in the same enteroendocrine cell has been reported in mammalian species. Egerod *et al*. (2012) reported coexpression of neurotensin with some other hormones, such as cholecystokinin, secretin and GLP-1, in the mouse intestine. Svendsen *et al*. (2015) demonstrated coexpression of neurotensin with GLP-1 in the rat small intestine. Recent study detected three types of EECs in the chicken ileum on the basis of the colocalizational pattern of GLP-1 with neurotensin (Nishimura *et al*., 2017). According to this study, cells containing both GLP-1 and neurotensin were more frequently observed than cells containing either hormone in the chicken ileum. Moreover, GLP-1-immunoreactive cell also decreased its density with the decrease of protein intake (Monir *et al*., 2014a). Judging from these findings and the present data, it is possible that EECs which contain both neurotensin and GLP-1 are sensitive to the dietary protein. But more systematic experiments are necessary for this possibility.

In the present experimental diets, cornstarch was included in an increased amount to maintain the constant energy density in the all groups (ME=2847-2850 kcal/kg DM).

Several studies on mammals indicated that carbohydrate influenced the secretion of neurotensin from the intestinal N cells in a dose-dependent manner (Dakka *et al*., 1993; Dumoulin *et al*., 1998; Kuhre *et al*., 2015). If there was any incentive effect of increased level of cornstarch on N cells frequencies and morphological features in the chicken small intestine could be appeared in the lower dietary CP groups. In the present results, however, such outcomes were not found in the lower CP groups. Thus, it indicates that cornstarch does not have stimulative influences on N cells in the chicken small intestine.

In conclusion, dietary protein level is used as an effective stimulator on neurotensin secretion from N cells in the chicken ileum.

Table 2
Compositions of experimental diets used in this study (g).

Composition	CP 18%	CP 9%	CP %	CP %
ISP (CP84%)	214.0	107.0	53.5	0.0
L-methionine	1.3	0.7	0.4	0.0
L-cystine	2.2	1.1	0.6	0.0
Cornstarch	493.1	599.1	652.1	705.2
Cellulose	196.4	199.1	200.4	201.8
Corn oil	30.0	30.0	30.0	30.0
Mineral mixture	58.5	58.5	58.5	58.5
Vitamin mixture	2.0	2.0	2.0	2.0
Choline chloride	1.5	1.5	1.5	1.5
Inositol	1.0	1.0	1.0	1.0

They were composed at the same energy density (metabolizable energy = 2850 kcal/kg) with the same protein source (ISP: isolated soybean protein)

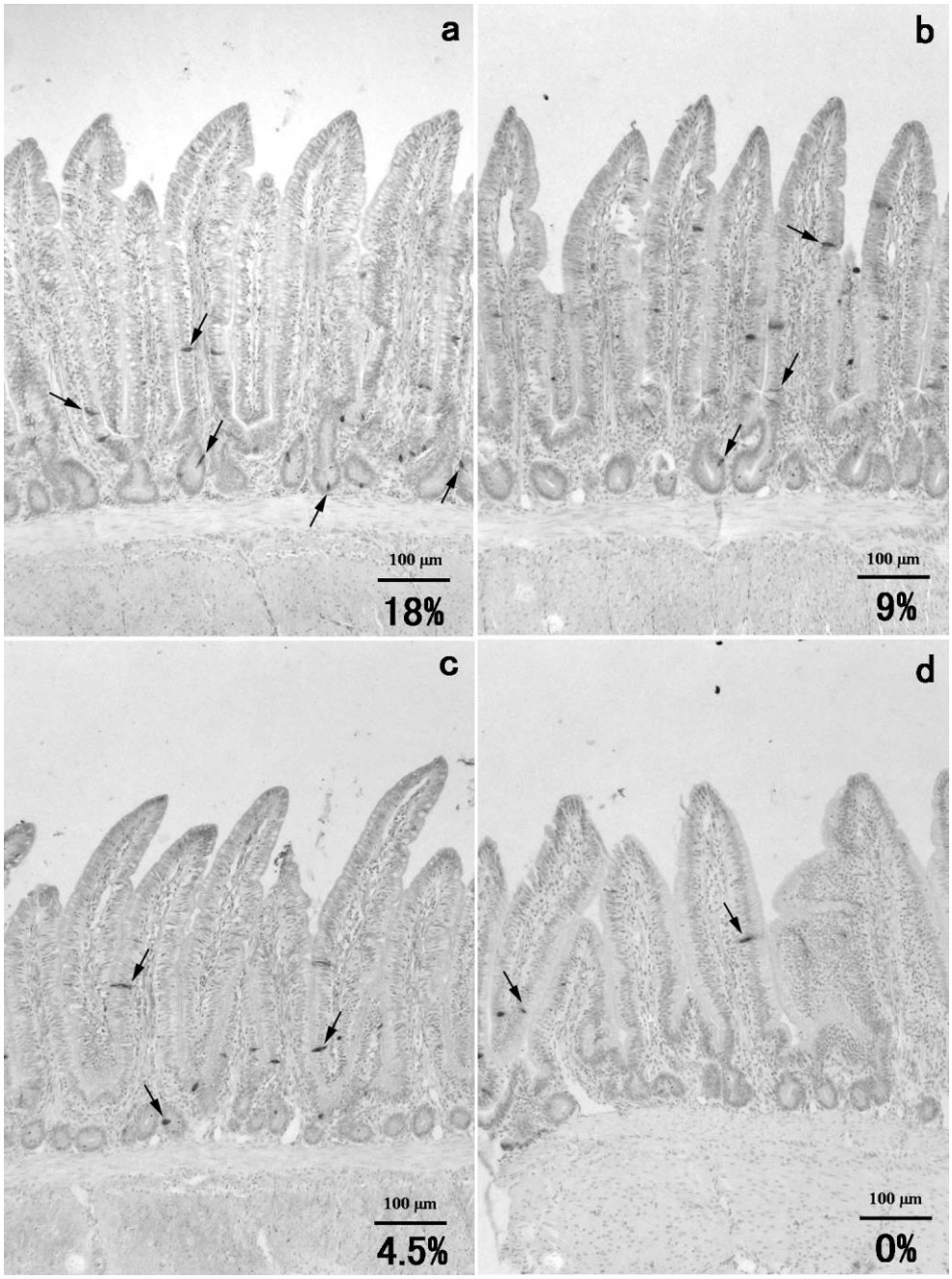

Fig. 5. Low magnification views showing the distributional pattern of neurotensin-immunoreactive cells (arrows) in the ileum from chickens fed with four different dietary protein level, CP18% (a), 9% (b), 4.5% (c) and 0% (d). Neurotensin-immunoreactive cells are distributed from crypt to higher part of villous epithelium in all groups, but their numbers are significantly decreased in CP4.5% and CP0% groups (c, d) compared with CP18% group (a).

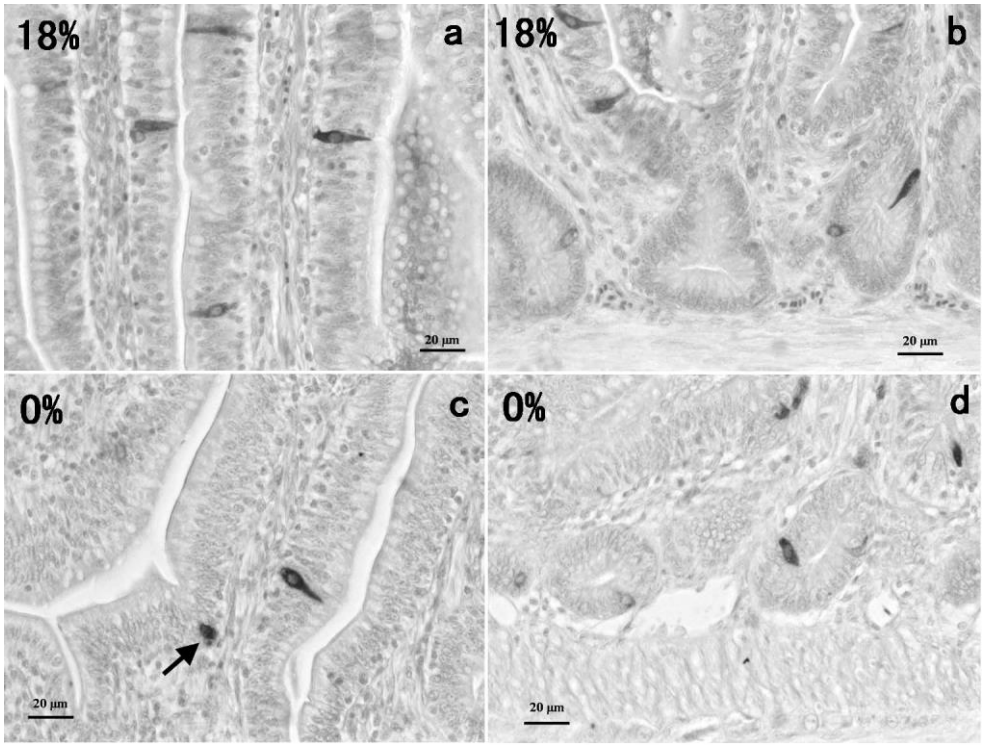

Fig. 6. High magnification view of neurotensin-immunoreactive cells in villus epithelium (a, c) and crypts (b, d) of ileum from chickens of CP 18% (a, b) and CP 0% (c, d) groups. Neurotensin-immunoreactive cells have a flask or spindle-like shape with a long cytoplasmic process reaching to the intestinal lumen, but round or oval cell showing immunoreactivity for neurotensin is detected in villous epithelium of CP 0% group (arrow).

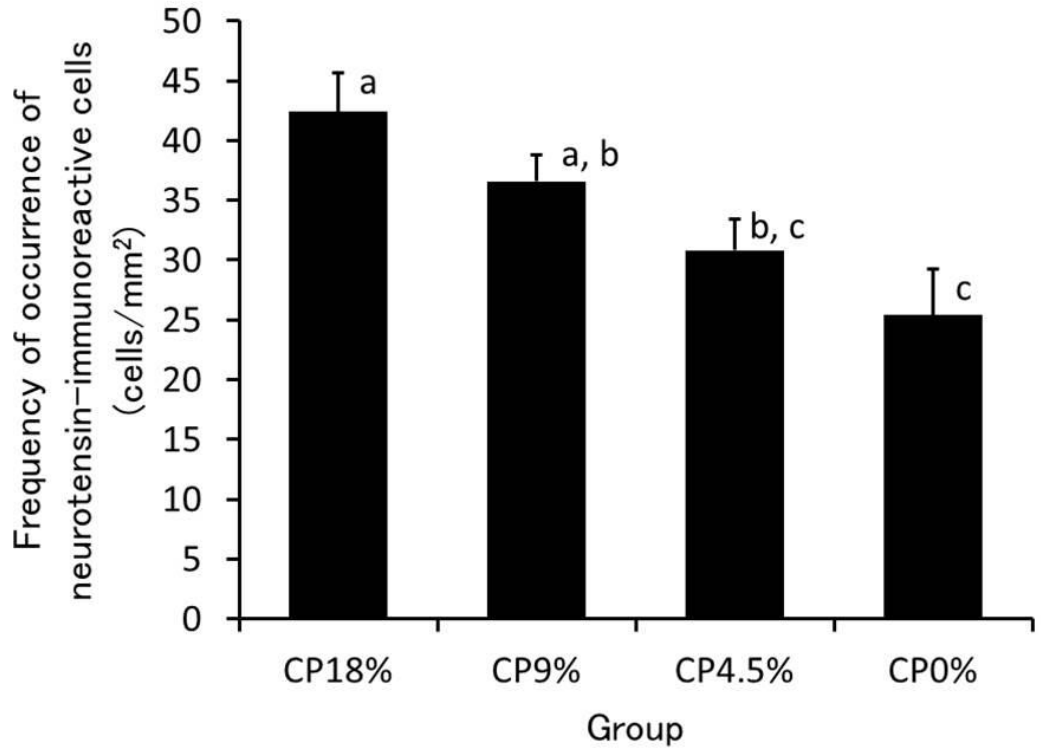

Fig. 7. Frequencies of occurrence of neurotensin-immunoreactive cells in ileum from chickens fed with four different dietary protein levels (CP 18%, 9%, 4.5% and 0%). Values are represented by the average number of neurotensin-immunoreactive cells per 1 mm^2 mucosa. There are significant differences between different letters (P<0.05, error bar: standard deviation).

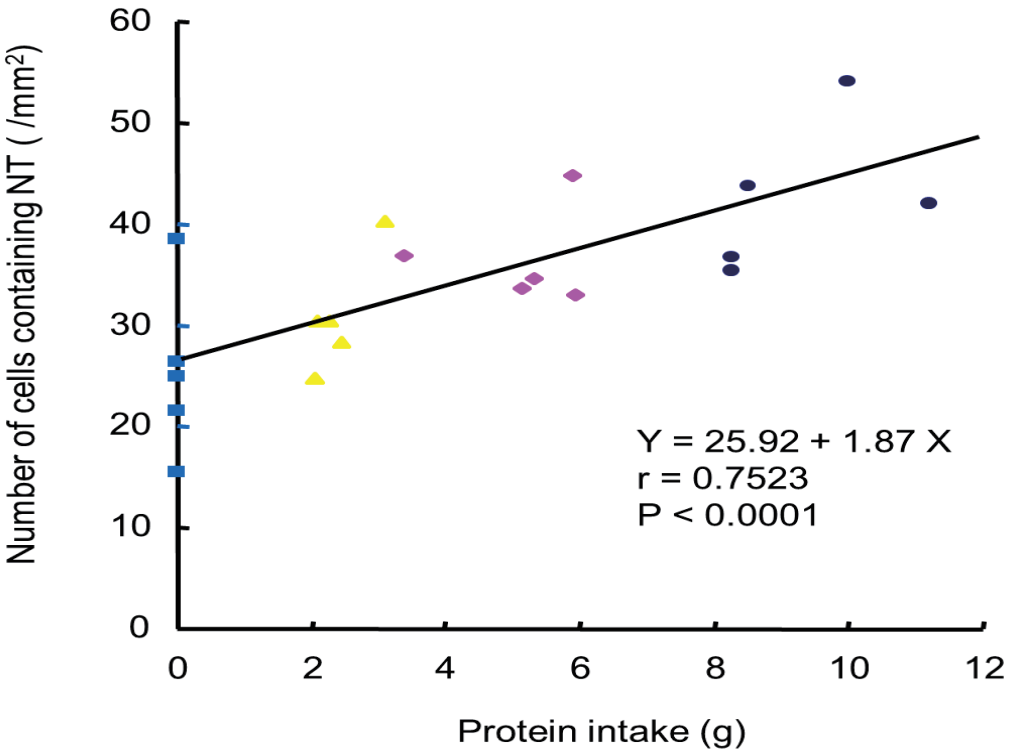

Fig. 8. Regression line of frequency of occurrence of neurotensin-immunoreactive cells (Y) on the daily protein intake (X). The equation of this line is Y=25.92+1.87X, *P*<0.0001.

III. 2. Summary

The purpose of this experiment was to evaluate the effect of dietary protein on Neurotensin (NT)-immunoreactive cells in the chicken ileum. Male White Leghorn chickens were used in this study. Chicken were divided into four groups with different dietary crude protein (CP) levels: 18 % (control), 9 %, 4.5 %, and 0 %, based on average body weight of five in each. Morphometric analysis and indirect immunohistochemistry techniques were employed to detect EECs. The findings demonstrated that the NT-immunoreactive cells exhibited a long cytoplasmic process in epithelium of villi in the control groups. This cytoplasmic process arrived to the intestinal lumen and could sense nutrients as a chemical signal. On the other hand, NT-immunoreactive cells had an oval or round-like shape in the lower CP groups, particularly in 0% CP group. Moreover, the frequency of NT-immunoreactive cells decreased while the level of CP in the diets was decreased and found significantly lower number of NT-immunoreactive cells in the lower CP groups, especially in 0% CP group. In addition, the NT-immunoreactive cells also increased with the CP inake increased because there was a significantly positive relation between daily CP intake and frequency of the NT-immunoreactive cells. Therefore, this present results suggest that dietary CP is one of the potent stimulator to activate the intestinal N cells in the chicken ileum.

Chapter IV

Dietary nutrient influences colocalization pattern of hormones in endocrine cells

IV. 1. Dietary carbohydrate influences the colocalization pattern of glucagon-like peptide-1 with neurotensin in the chicken ileum

IV. 1. 1. Introduction

Glucagon-like peptide (GLP)-1 is a 30 amino acid peptide derived from specific post-translational proteolytic cleavage of proglucagon (PG) precursor and secreted from enteroendocrine L cells (Baggio and Drucker, 2007; Karhunen *et al*., 2008). This peptide plays a vital role in regulating numerous physiological functions in the gastroenteropancreatic (GEP) system. Some of these functions include enhancing glucose-dependent insulin release and inhibiting intestinal motility (Nauck, 1998; Drucker, 2006; Holst, 2007). Several studies in mammals have shown that L cells are distributed in both large and small intestines (Damholt *et al*., 1999; Cho *et al*., 2015), but they are predominantly found in the jejunum and ileum in chicken, with the highest frequency in the distal ileum (Hiramatsu *et al*., 2003; Hiramatsu *et al*., 2005). Our previous studies demonstrated that restricted feeding and dietary nutrients influence the density of GLP-1-immunoreactive cells in the small intestine of chicken (Monir *et al*., 2014a; Monir *et al*., 2014b; Nishimura *et al*., 2015).

Neurotensin (NT) is a 13 amino acid peptide derived from neurotensin precursor (NTP) protein, which consists of 169-170 amino acid (Kislauskis *et al*., 1988). This peptide was isolated from bovine hypothalamus extract for the first time and found in N cells of avian and mammalian intestines (Carraway and Leeman, 1973; Carraway and Ferris, 1983). The C-terminal of this peptide is highly conserved in various species, suggesting that it exerts certain biological activities. NT is secreted from intestinal N cells and plays various roles in the gastrointestinal tract, such as regulating gastric and intestinal motility and inducting pancreatic and biliary secretion (Thor and Rosell, 1986;

Gui *et al*., 2001; Zhao and Pothoulakis, 2006). N cells were found to be present along the whole of the intestinal tract of chickens (Atoji *et al*., 1994), with higher frequency in the ileum (Nishimura *et al*., 2017).

Many studies have provided evidence concerning the colocalization of plural hormones in an enteroendocrine cell (EEC) (Schonhoff *et al*., 2004; Cho *et al*., 2014; Cho *et al*., 2015; Svendsen *et al*., 2015; Grunddal *et al*., 2016). This phenomenon contradicts the traditional "one cell, one hormone" theory in endocrinology. Recent studies have described the intricate colocalization pattern of GEP peptides in various species (Cho *et al*., 2014; Cho *et al*., 2015; Grunddal *et al*., 2016). Another study found that GLP-1-immunoreactive cells overlapped with NT-immunoreactive ones to various extents in the mouse small intestine (Svendsen *et al*., 2015). Pronounced colocalization between GLP-1 and NT was identified in the mammalian ileum (Schonhoff *et al*., 2004). In the chicken ileum, GLP-1 colocalized with NT in the same EECs at high rates (Nishimura *et al*., 2017).

These two peptides are released from their respective EECs in response to the ingestion of food. Many in vitro and in vivo studies have described that nutrients such as carbohydrates, glucose, protein, fat, and lipids influence the release of GLP-1 and NT in mammalian and avian species (Mansour *et al*., 2013; Monir *et al*., 2014a, Svendsen *et al*., 2015; Svendsen and Holst, 2016). Moreover, various secretagogues such as hormonal, neuronal, and metabolic ones were shown to stimulate the cosecretion of GLP-1, PYY, and NT, whereas this was shown to occur more for GLP-1 and NT than for PYY in the rat ileum (Grunddal *et al*., 2016). Svendsen *et al*. (2015) reported that the luminal infusion of nutrients could influence the corelease of different hormones in the small intestine of rat. These studies indicated that different stimuli could influence the patterns of colocalization of various peptides in single EECs in the intestine. Since various

stimulators influence hormone co-secretion, altering these EECs could potentially be achieved by changing the dietary nutrient intake.

However, no evidence has been presented showing that specific nutrients could influence the patterns of colocalization of GLP-1 with NT. To date, no data has been published regarding the influence of dietary CHO on the colocalization of GLP-1 with NT in chickens. Therefore, I designed the present study to reveal the influences of dietary level of CHO on the degree of colocalization of GLP-1 with NT in the distal ileum of chicken.

IV. 1. 2. Materials and Methods

1. 2. 1. *Experimental animals and feeding management*

The animal experimental protocol of the present study was reviewed by the Committee for Animal Experiments and finally approved by the president of Shinshu University (Approval Number 300090).

Male White Leghorn chickens at 6 wk. of age (n =15) were grouped into a control group and two experimental groups (low-CHO and CHO-free), containing five birds each and matched for body weight. The control diet shown in Table 3 was supplied for 3 days as habituation to the experimental feeding setup in separate cages. After completing habituation, the experimental diets shown in Table 3 were fed for 7 days under controlled light conditions (12 hr light: 12 hr dark). Chickens could access feed and water freely during the experimental period. Daily feed intake and body weight of each chicken were measured at the same clock time during the experimental period.

The same CHO source (corn starch) was used in the experimental diets like that of the control group. CHO levels of feeds for the low-CHO and CHO-free groups were

12.5% and 0% of the control feed, respectively. To maintain the same energy level [metabolizable energy (ME) = 2,847-2,850 kcal/kg dry matter (DM)], the inclusion level of fat (corn oil) was increased in the experimental groups. The ME requirement (2,850 kcal/kg ME) of the diets followed the Japanese Feeding Standard for Poultry (NARO, 2011).

1. 2. 2. *Tissue sampling*

After completing the feeding regime, the chickens were sacrificed by decapitation under anesthesia with sodium pentobarbital. Distal ileum between ceca about 2 cm long was immediately dissected out for the tissue sample from each chicken. Intestinal contents were gently washed out with 0.75% NaCl solution, and immersed in Bouin's fluid at room temperature for 24 hr. Paraffin sections were cut at 5 μm thickness in the conventional manner and used in the subsequent analyses.

1. 2. 3. *Double immunofluorescence staining for GLP-1 and NT*

Double immunofluorescence staining was carried out to detect the colocalization of GLP-1- and NT-immunoreactivity in the distal ileum. Paraffin sections treated with 10% normal goat serum for 20 min were incubated with a mouse monoclonal antibody against synthetic human GLP-1 (aa 7-36) (A6104.1, Immunodiagnostik, Bensheim, Germany; diluted to 1:2,000) for 24 hr. After washing with phosphate buffered saline (PBS), sections were incubated with a rabbit anti-NT serum (AB5496; Millipore, Temecula, CA, USA; 1:500) for 24 hr. After three washes with PBS, sections were incubated with a cocktail of goat anti-mouse IgG labeled with DyLight 488 (611-741-127; Rockland Immunochemicals, Gillbertsville, PA, USA; 1:300) and goat anti-rabbit IgG labeled with DyLight 549 (611-700-127; Rockland Immunochemicals, Gillbertsville, PA, USA;

1:300) as secondary antibodies for 3 hr. After coverslipping with an aqueous mounting medium (PermaFluor; Thermo Fisher Scientific, Fremont, CA, USA), preparations were observed and photographed under a computerized fluorescent microscope (AxioImager A1, Zeiss, Göttingen, Germany). All incubation steps were performed in a moisture chamber at room temperature. Specificities of the primary antibodies used in this study were well documented by the manufacturers and our previous study (Nishimura *et al*., 2017).

Morphometric analysis was carried out to evaluate the amount of GLP-1 and NT colocalization in EECs, in accordance with a previously described manner (Svendsen *et al*., 2015). The mucosal epithelium was divided into three parts, crypts, and bottom and middle parts of villi. Twenty areas were randomly selected from each epithelial part of each chicken to count immunoreactive cells. One hundred parts of each epithelial part in total were observed from five chickens in each group.

1. 2. 4. *In situ hybridization for proglucagon (PG) mRNA and NT precursor (NTP) mRNA*

Mirror sections at 5 μm thickness were made from each paraffin block, in accordance with a previously described manner (Nishimura *et al*., 2017). *In situ* hybridization for mRNAs of PG and NTP was carried out using a commercial kit (IsHyb In Situ Hybridization Kit; Biochain Institute, Newark, CA, USA) following the manufacturer's instructions. Sections were incubated with an anti-DIG antibody diluted in alkaline-phosphatase solution (1:500), and mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate was applied to visualize the conjugated probe under a light microscope. All oligonucleotide probes were commercially (BEX, Tokyo, Japan) synthesized according to the PG sequence (Richards and McMurtry, 2008) and the NTP

sequence (Tanaka *et al*., 2013). Sequences of antisense and sense probes of PG and NTP were indicated in Table 4.

Subsequently, sections were incubated with rabbit anti-NT serum as a primary antibody for 24 hr. After three washes with PBS, sections were treated with goat anti-rabbit IgG serum labeled with Dylight-549 as a secondary antibody for 3 hr. The complement of this mirror section was hybridized with NTP antisense probe and then incubated with rabbit anti-GLP-1 serum for 24 hr, and subsequently incubated with donkey anti-rabbit IgG serum labeled with Dylight-488 for 3 hr. All incubations were carried out in a moisture chamber at room temperature. Photographs were taken under a microscope (AxioImager A1; Zeiss) after coverslipping sections with an aqueous mounting medium. Sense probes were applied as a negative control.

1. 2. 5. *Statistical analysis*

All data about ratio of colocalization of GLP-1 and NT in each epithelial part of each group are presented as mean ± standard error of the mean. In order to compare the three groups statistical analysis was carried out by one-way analysis of variance. Tukey's test was applied to determine the significance of differences among the means. Data were considered significant at $P < 0.05$. All statistical analysis was performed using statistical analysis software (SAS, Inst. Inc., Cary, NC, USA).

IV. 1. 3. Results

Two experimental groups showed lower daily feed intake and weight gain compared to those in the control group. However, no significant differences in these parameters were observed among three groups.

1. 3. 1. *Effects of dietary CHO on the colocalization of GLP-1 with NT*

Double immunofluorescence staining revealed three types of endocrine cell based on the combination of immunoreactivity against GLP-1 and NT antisera in the distal ileum of all groups. The first type of endocrine cell showed immunoreactivity for both GLP-1 and NT (GLP-1+/NT+) (Fig. 9). The second and third ones showed either GLP-1- (GLP-1+/NT−) or NT-immunoreactivity (GLP-1−/NT+) (Fig. 10). All types of endocrine cell were distributed throughout the distal ileum of chickens. There were no apparent differences in the distribution pattern of these cells among the three groups. However, differences were observed in the shape of GLP-1+/NT+ cells between the control and two experimental groups. Specifically, most GLP-1+/NT+ cells in the control group had a spindle-like or flask-like shape with a long cytoplasmic process reaching the intestinal lumen (Fig. 9, arrows). In contrast, the majority of GLP-1+/NT+ cells in the two experimental groups, especially the CHO-free group, were round or oval in shape (Fig. 9, arrowheads).

The ratios of each cell type in each epithelial part are summarized in Fig. 11. The ratio of GLP-1+/NT+ cells (Fig. 11, yellow columns) was significantly higher ($P< 0.05$) in the control group than in the two experimental groups and showed the trend of being lower as dietary CHO level decreased. This trend was identified in each epithelial part. On the other hand, the ratio of GLP-1−/NT+ cells (Fig. 11, green columns) was highest in the CHO-free group and increased with decreasing dietary CHO level at the middle and bottom parts of villi. This ratio was significantly higher ($P< 0.05$) in the CHO-free group than in the control group at the bottom part of villi and crypts. The ratio of GLP-1+/NT- cells (Fig. 11, blue columns) was lowest in the control group at each epithelial

part. There was no significant difference in the ratio of GLP-1+/NT- cells between the two experimental groups at each epithelial part.

1. 3. 2. *Effects of dietary CHO level on proglucagon (PG) and NT precursor (NTP) mRNA signals*

PG and NTP mRNA signals were detected in the distal ileum of all groups by *in situ* hybridization (Fig. 12). Cells expressing PG mRNA signal were primarily localized in the epithelium of the bottom part of villi and crypts. Cells expressing NTP mRNA signal were observed from crypts to the middle part of villi. No clear differences were observed in the distribution patterns of cells expressing PG and NTP mRNA signals among the three groups. However, the relative frequency of cells expressing PG and NTP mRNA signals was apparently higher in the control group than two experimental groups. A decreasing trend of the frequency of cells expressing PG and NTP mRNA signals was observed with the decrease of dietary CHO level (Table 5).

The majority of endocrine cells expressing both PG mRNA signal and NT immunoreactivity (Fig. 13a, a') were observed in villus epithelium and crypts of the control group. These cells also showed NTP mRNA signal and GLP-1 immunoreactivity on the complement of this mirror section (Fig. 13b, b'). Endocrine cells showing immunoreactivity for both GLP-1 (Fig. 13d', f') and NT (Fig. 13c', e') peptides with neither PG (Fig. 13c, e) nor NTP (Fig. 13d, f) mRNA signal were frequently detected in the experimental groups, especially in the CHO-free group.

IV. 1. 4. Discussion

The present study revealed that the dietary CHO level influences not only the colocalization pattern of GLP-1 with NT, but also the morphology of endocrine cells in

the chicken ileum. EECs of the open type have a spindle-like or flask-like shape with a long cytoplasmic process reaching the intestinal lumen. One previous study using electron microscopy demonstrated that chicken intestinal L cells were the open type of endocrine cell (Nishimura *et al*., 2013). Different types of receptor are well known to be present on the surface of microvilli of open type endocrine cells, and thus microvilli function as receivers of chemical signals in the intestinal lumen (Breer *et al*., 2012). Moreover, a study revealed that L cells had glucose-sensing receptors on their apical surface (Shima *et al*., 1990). Therefore, GLP-1+/NT+ cells having a long cytoplasmic process may monitor the glucose level in the chicken intestinal lumen.

In the present study, endocrine cells exhibiting GLP-1 and/or NT immunoreactivity in the control group showed the same shape as the open type, but those in the experimental groups, especially the CHO-free group, were round or oval in shape and lacked a cytoplasmic process. Endocrine cells with a round or oval shape were frequently observed in the epithelium of the fasted chicken ileum (our unpublished observation). Moreover, I recently provided evidence that dietary CHO affected the proliferation of epithelial cells in the chicken ileum (Salahuddin *et al*., 2021). These findings suggest that dietary CHO is an essential nutrient for the maintenance of EECs in the chicken ileum. Zhou *et al.* (2018) demonstrated that glucose stimulated the proliferation of mouse intestinal crypt. Moreover, Higashizono *et al.* (2019) revealed that CHO supplementation preserved the gut morphology of mice after short-term fasting. These studies indicate the relationship of dietary CHO to the maintenance of intestinal epithelium and support my suggestion mentioned above.

Many studies have reported that plural hormones colocalize in the same EEC in mammalian species. Immunohistochemical studies demonstrated the colocalization of

GLP-1 with other peptide hormones, such as cholecystokinin, glucose-dependent insulinotropic polypeptide, peptide YY and secretin (Mortensen *et al*., 2003; Egerod *et al.*, 2012; Svendsen *et al.,* 2015; Fothergill *et al*., 2017). Recent previous study revealed that GLP-1 colocalized with GLP-2 in the same secretory granules of endocrine cells of the chicken ileum (Nishimura *et al*., 2013). In addition, another recent study performed in 7-day-old chicks also demonstrated the colocalization of GLP-1 with NT in the same EEC (Nishimura *et al*., 2017). Thus, the colocalization of plural hormones in the same EEC is a common phenomenon even in the chicken small intestine. This same study also observed three types of EEC on the basis of the combination of immunoreactivity against GLP-1 and NT antisera (Nishimura *et al*., 2017). The same results regarding the cell type were obtained in this study, that is to say, dietary CHO level did not alter cell types based on the combination of immunoreactivity against GLP-1 and NT. However, the ratio of each cell type was altered by the dietary CHO level. Luminal nutrients are well known to change the density of EECs. Previous studies indicated that dietary protein and amino acids altered the density of GLP-1-immunoreactive cells in the chicken ileum (Monir *et al*., 2014a; Nishimura *et al*., 2015). In this study, GLP-1+/NT+ cells displayed a particular changes in their ratio, being significantly reduced with the reduction of dietary CHO level. This suggests that GLP-1+/NT+ cells are more sensitive to carbohydrate than cells including GLP-1 or NT only. Plural hormones costored in the same EEC are presumably cosecreted and exert various physiological actions, such as regulating food intake and digestion (Fothergill *et al*., 2018). For example, 5-hydroxytryptamine, secretin and cholecystokinin are costored in the same EEC and these hormones control digestion in the intestine by regulating motility and secretion of digestive agents (Fothergill *et al*., 2018). It is expected that GLP-1 and NT are co-secreted from L cells in response to CHO

ingestion and react on the regulation of intestinal motility, because this physiological function of these hormones is reported in mammalian species (Thor and Rosell, 1986; Zhao *et al*., 2006). Further systematic studies are necessary for a full understanding of this matter.

The *in situ* hybridization technique applied in this study demonstrated that the densities of endocrine cells exhibiting PG and NTP mRNA signals were reduced in the CHO-free group. Moreover, most cells showing immunoreactivity for GLP-1 and NT antisera were deficient in PG and NTP mRNA signals, respectively, in the experimental groups, especially in the CHO-free group. These results indicate that dietary CHO level may regulate PG and NTP mRNA transcription, which might influence the colocalization pattern of these two peptides in the same endocrine cells of the distal ileum. Several studies demonstrated that glucose was the most potent stimulator regulating the transcription of proglucagon in GLUTag, GLP-1-secreting, cell culture (Daoudi *et al*., 2011; Puddu *et al*., 2014). Another study showed that a diet including a high level of glucose could upregulate the mRNA transcription of neurotensin in the jejunum of mice (Du *et al*., 2010). Therefore, it is possible that the low dietary CHO level suppressed the transcription of PG and NTP mRNA, and the ratio of GLP-1+/NT+ cells was reduced by this suppression in the distal ileum of chicken.

IV. 1. 5. Conclusion

Specific nutrients could influence the secretion of different hormones by altering the density of EECs in order to perform various functions in the gut. The present findings suggest that a low dietary CHO level reduces the occurrence of GLP-1+/NT+ cells in the distal ileum of chicken.

Table 3
Composition of the control and experimental diets used in the present study.

Composition	Control	Low-CHO	CHO-free
Isolated soybean protein	217.4	217.4	217.4
L-Cysteine	0.7	0.7	0.7
L-Methionine	0.9	0.9	0.9
L-Threonine	0.4	0.4	0.4
Cornstarch	491.4	61.4	0.0
Cellulose	194.7	449.3	485.7
Corn oil	30.0	205.4	230.4
Mineral mixture	60.0	60.0	60.0
Vitamin mixture	2.0	2.0	2.0
Choline chloride	1.5	1.5	1.5
Inositol	1.0	1.0	1.0

The same carbohydrate (CHO) source (cornstarch) was added to maintain the same energy density (metabolizable energy = 2850 kcal/kg) of the diets. Unit=g/kg.

Table 4
Sequences of antisense and sense probes used for *in situ* hybridization.

Protein Accession No.	Sequence of antisense probe Sequence of sense probe	Reference
Proglucagon S78477	5'-GCTGTAGTCACTGGTGAATGTGCCTTGTGAATGACGCTTTA-3' 5'-TAAAGCGTCATTCACAAGGCACATTCACCAGTGACTACAGC-3'	[Richards and McMurtry, 2008]
Neurotensin precursor XM_416126	5'-TCAAATGCGTCTTGCTGAAGTAACTCCCAGTGCTGAAAGG-3' 5'-CCTTTCAGCACTGGGAGTTACTTCAGCAAGACGCATTTGA-3'	[Tanaka *et al.*, 2013]

Table 5
Relative frequency of cells expressing proglucagon (PG) and neurotensin precursor (NTP) mRNA signals in the distal ileum of chicken.

	Control	Low-CHO	CHO-free
PG	+ +	+	±
NTP	+ +	+	±

+ + : moderate, +: few, ±: rarely, -: none, PG: proglucagon, NTP: neurotensin precursor, CHO: carbohydrate.

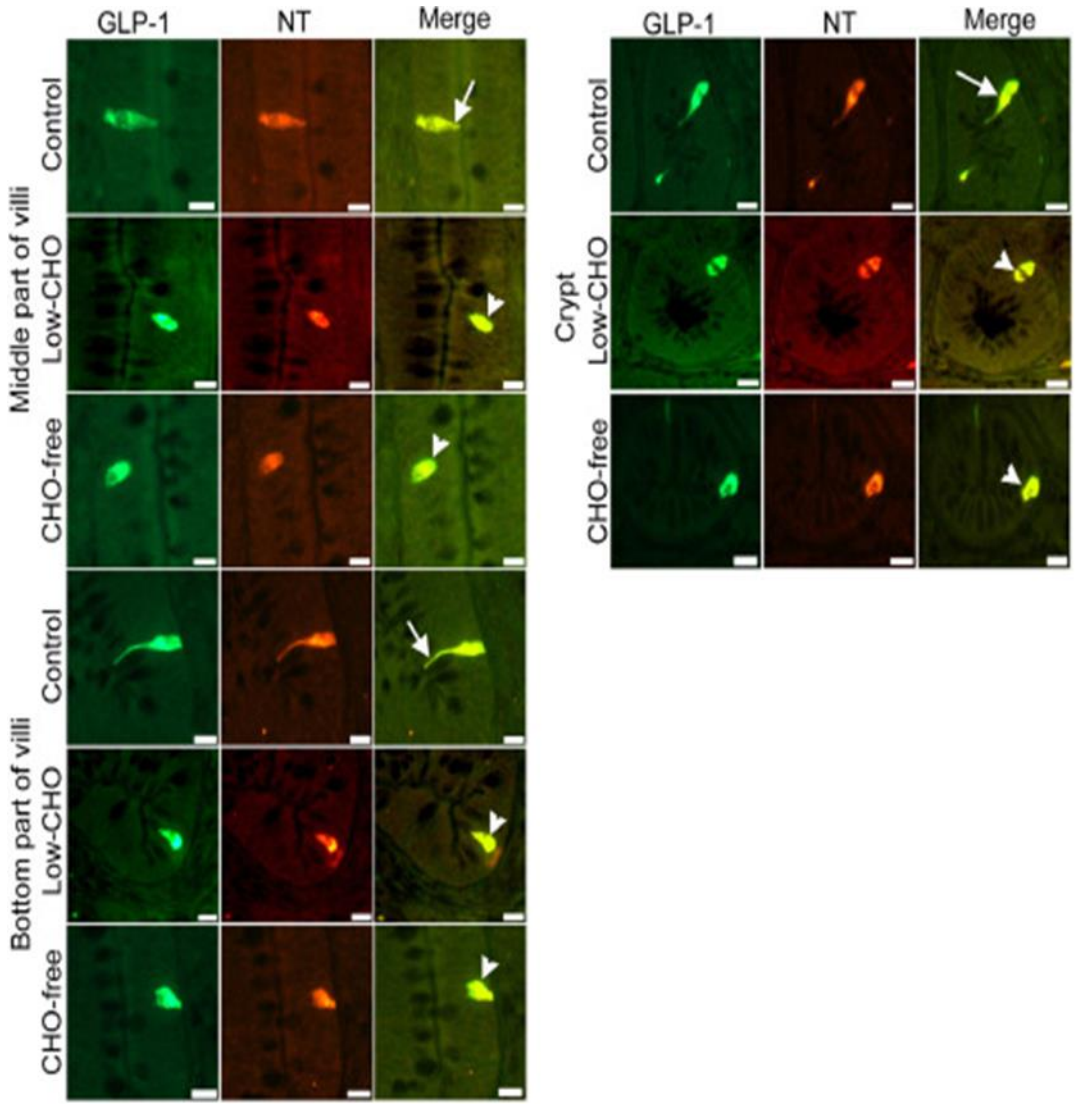

Fig. 9. Photomicrographs of enteroendocrine cells showing both glucagon-like peptide (GLP)-1 and neurotensin (NT) immunoreactivity (GLP-1+/NT+) in the epithelium of middle and bottom parts of villi and crypts in the distal ileum of the control, low-CHO, and CHO-free groups. Double immunofluorescence staining was carried out to detect enteroendocrine cells showing immunoreactivity for GLP-1 and NT antisera. Arrows indicate a long cytoplasmic process reaching the intestinal lumen. Arrowheads indicate GLP-1+/NT+ cells in a round or oval shape. Scale bars:10μm.

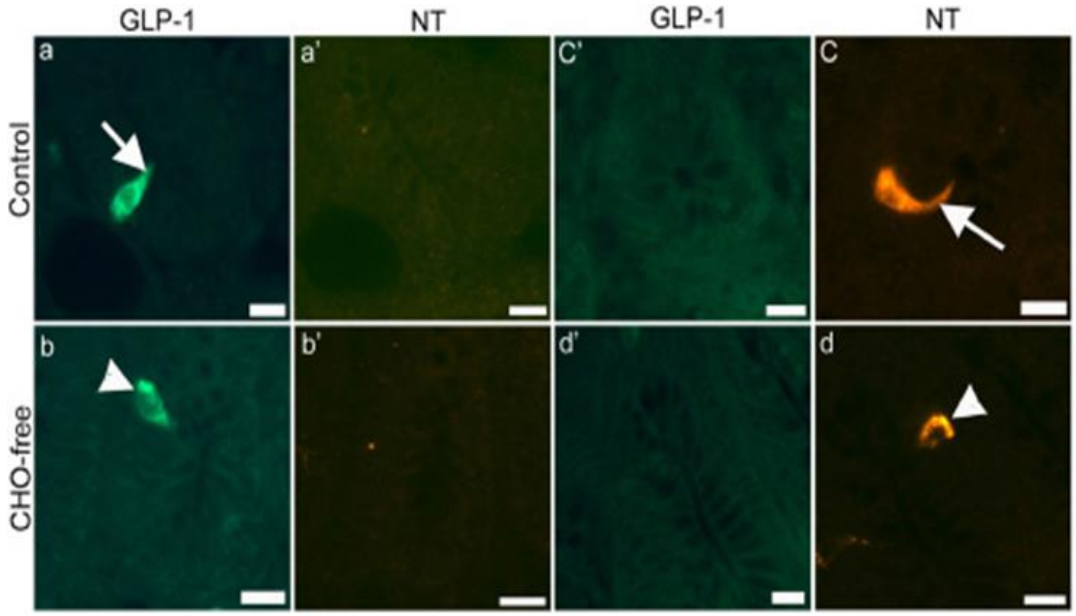

Fig. 10. Photomicrographs showing GLP-1+/NT− (a, b) and GLP-1−/NT+ (c, d) cells in crypts in the distal ileum of the control (a, c) and the CHO-free (b, d) groups. Scale bars:10μm

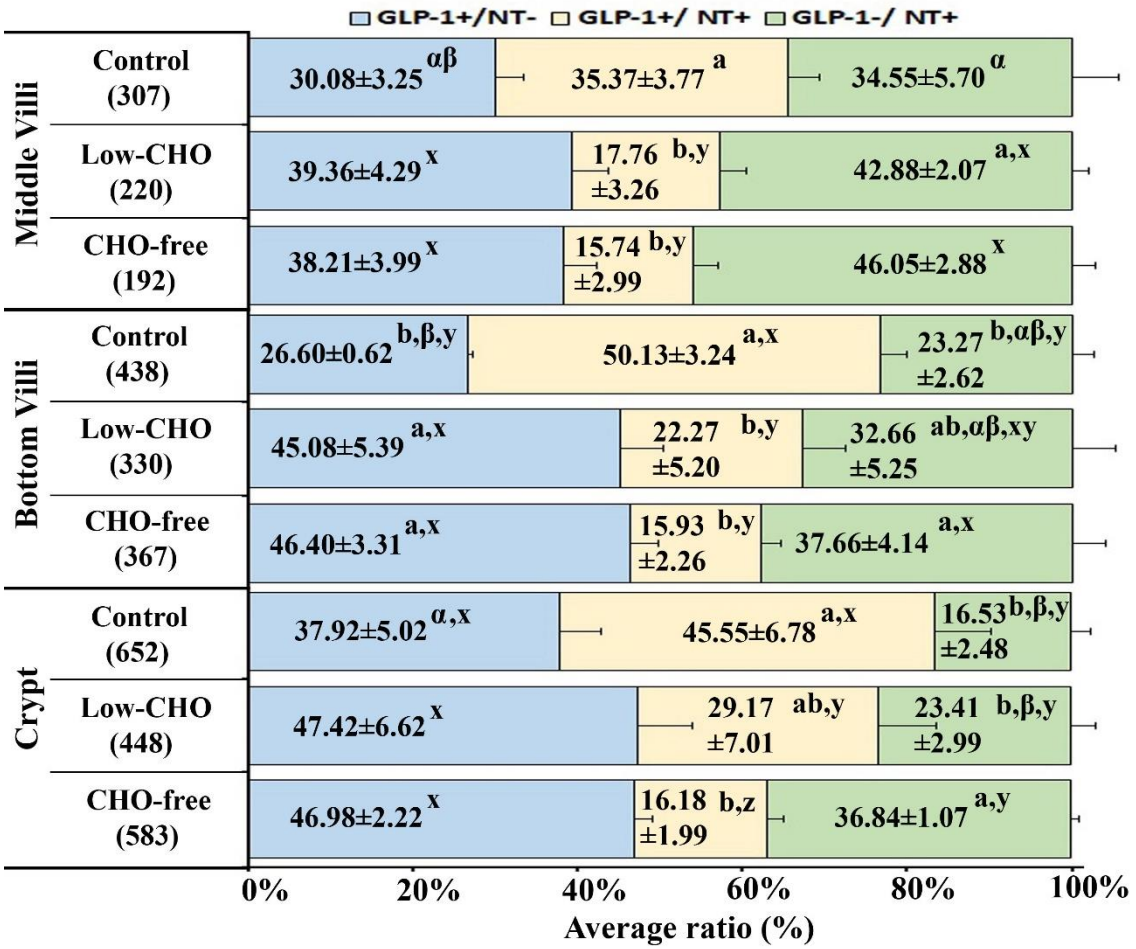

Fig. 11. Average ratios of enteroendocrine cells detected by double immunofluorescence staining using glucagon-like peptide-1 (GLP-1) and neurotensin (NT) antisera in three epithelial parts, middle and bottom parts of villi and crypts of distal ileum, of the control, low-CHO, and CHO-free groups. Three types of endocrine cell are revealed on the basis of the combination of immunoreactivity against GLP-1 and NT antisera. Blue, yellow and green columns represent percentages of cells showing immunoreactivity for only GLP-1 (GLP-1+/NT−), both GLP-1 and NT (GLP-1+/NT+), and only NT (GLP-1−/NT+), respectively. Number in each column represents the average percentage of each cell type and standard error of the mean (mean ± SEM). a, b: There is a significant difference between values with different subscripts at $P < 0.05$ in each cell type in each epithelial part. x, y, z: There is a significant difference between values with different subscripts at $P < 0.05$ in each group in each epithelial part. α, β: There is a significant difference between values with different subscripts at $P < 0.05$ parts in each cell type in each group. Error bars: Standard error of the mean (SEM).

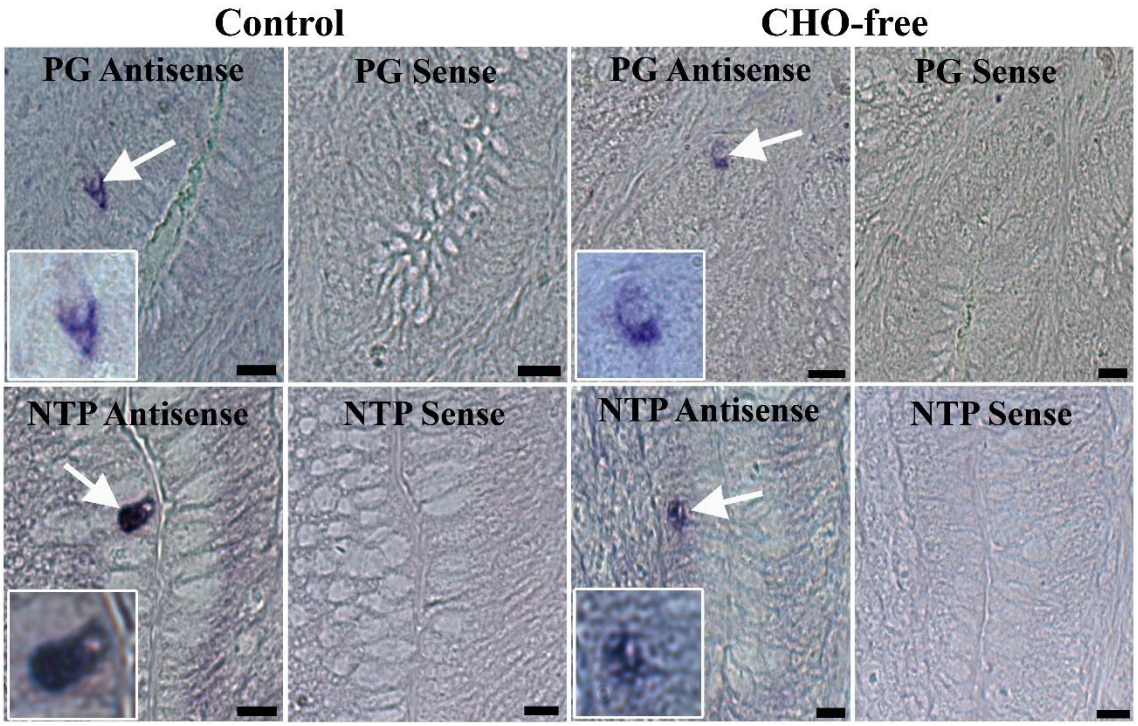

Fig. 12. Photomicrographs of cells expressing mRNA signals of proglucagon (PG) and neurotensin precursor (NTP) in the distal ileum of the control and CHO-free groups. Cells expressing PG and NTP mRNA signals are detected by *in situ* hybridization. Sections incubated with PG antisense-oligoprobe show the specific PG mRNA signal (arrows in upper panel), but those incubated with PG sense-oligoprobe no signal. Sections incubated with NTP antisense-oligoprobe show the specific NTP mRNA signal (arrows in bottom panel), but those incubated with NTP sense-oligoprobe no signal (bottom panel). Insets: High magnification views of cells expressing PG and NTP mRNA indicated by arrows. Scale bars: 10μm.

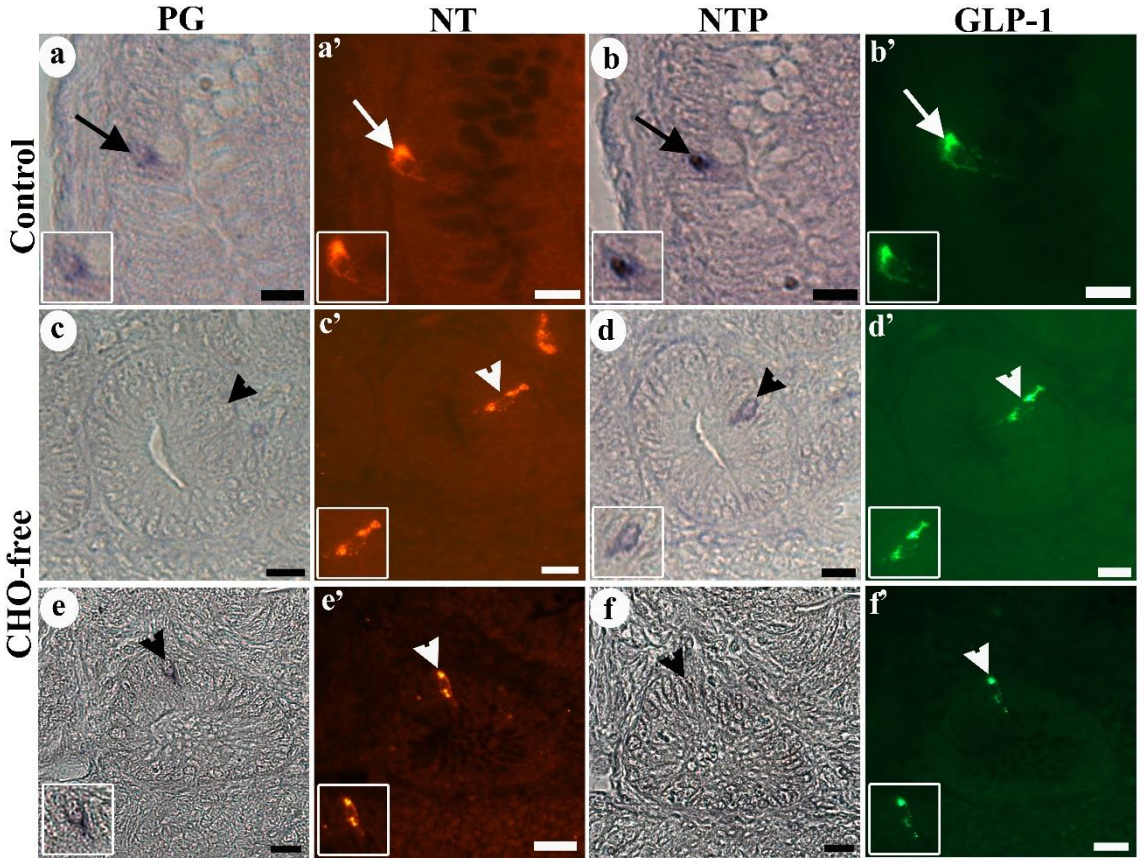

Fig. 13. Photomicrographs of cells expressing mRNA signals of proglucagon (PG) (a and e) and neurotensin precursor (NTP) (b and d). Cells expressing PG and NTP mRNA signals in the distal ileum were identified by *in situ* hybridization and those with immunoreactivity for neurotensin (NT) (a', c' and e') and glucagon-like peptide-1 (GLP-1) (b', d' and f') were identified by indirect immunofluorescence staining. Photomicrographs showing NTP mRNA (b, d, and f) and GLP-1 peptide (b', d', and f') are digitally reversed. Arrows in the top panels denote a cell showing PG and NTP mRNA signals and immunoreactivity for NT and GLP-1. Arrowheads in the middle panels denote a cell showing an NTP mRNA signal but not a PG mRNA signal with immunoreactivity for both NT and GLP-1. Arrowheads in the bottom panels denote a cell showing PG mRNA signal but not NTP mRNA signal with immunoreactivity for both NT and GLP-1. Insets: High magnification view of cells expressing PG and NTP mRNA and immunoreactivity for NT and GLP-1 indicated by arrows and arrowheads. Scale bars: 10 μm

IV. 2. Summary

This experiment was carried out to uncover the effects of dietary CHO on the colocalization pattern of GLP-1 with NT in the chicken distal ileum. Chicks were divided into a control and two treatment groups (Low-CHO and CHO-free groups contain 12.5% and 0% CHO of the control group) based on average body weight of five in each. Morphometric analysis, double immunoflurescenc staining, and in *situ* hybridization techniques were employed to detect colocalized EECs in order to investigate the influence of dietary CHO on these cells. Three types of EEC, GLP-1+/NT+, GLP-1+/NT− and GLP-1−/NT+, were demonstrated in this study. The control group's GLP-1+/NT+ cells were spindle-shaped with a long cytoplasmic process, whereas the experimental group's cells were round and lacked a cytoplasmic process. The ratio of GLP-1+/NT+ cells in the two experimental groups was considerably lower than in the control group. In both experimental groups, the ratio of GLP-1+/NT+ cells was substantially lower than the ratio of GLP-1+/NT and GLP-1/NT+ cells. In the experimental groups, the majority of cells immunoreactive for GLP-1 and NT antisera lacked proglucagon (PG) and NT precursor (NTP) mRNA signals. The number of EECs expressing PG and NTP mRNA signals tended to decrease as dietary CHO levels were decreased. These findings suggest that dietary CHO may play a substantial role in regulating the pattern of GLP-1 and NT colocalization in the same cells in the chicken ileum.

Chapter V

General Conclusion

General Conclusion

The intestine is the primary site of nutritional intake in chickens. The small intestine is a tube like structure and its inner epithelium part is folded into a fingerlike structure bordered by a single-layer columnar cells called villi. Villus is a histological component that is very important in this segment (Ragaa and Korany, 2016). The intestinal villi are involved in the last step of nutrient digestion and absorption. Consequently, the villus structure is essential for the effectiveness of the digestive and absorptive processes in the small intestine of birds. The epithelial enterocytes (absorptive cells) are covered with tiny protrusions known as microvilli, collectively referred to as brush broader, which significantly enhance the mucosal absorptive surface area by around 9 to 16 times (Helander and Fändriks, 2014). These microvilli reside in various types of nutrient transporters, channels and receptors for facilitating digestion and absorption. Microvilli are constituted of structural proteins, such as β-actin, as well as a variety of bundling proteins.Villin is a key β-actin bundling protein, and it is one of the most abundant proteins in the microvilli. Interestingly, the shape and size of the microvilli is not constant (Lecount and Grey, 1972; Craig and Powell, 1980; Stidwill *et al*., 1984). As a consequence, the structural characteristics of microvilli have been extremely variable depending on the condition of the actin and villin proteins. Several in vitro and in vivo investigations discovered that changes in actin microfilaments and villin levels affected the cytoskeleton of the microvillar core, which in turn impacted the structural characteristics of the microvilli. (Stidwill and Burgess, 1986; Friederich *et al*., 1989, 1999; Costa de Beauregard *et al*., 1995; Revenu *et al.,* 2012;). As a consequence, the free cell surface area may be altered, which may have an impact on the digestion and absorption of nutrients. However, a considerable amount of research demonstrated a

relationship between diet and intestinal mucosal integrity maintenance. In investigations, it has been shown that the presence of digested nutrients in the small intestinal lumen is related to architectural and physiological modifications of the mucosa (Yamauchi, 2002; Incharoen *et al*., 2010). Additionally, numerous studies have shown that carbohydrate, fructooligosaccharides, and mannan-oligosaccharide enhanced the free cell surface area for facilitating optimum nutrients digestion and absorbtion (Cheled-shovel *et al*., 2011; Shang *et al*., 2015; Zamani *et al*., 2018; Higashizono *et al*., 2019).

EECs are a diverse group of cells found throughout the GI tract that are defined by their capacity to retain and release hormones. Although EECs account for less than 1% of total epithelial cells, they together constitute the largest endocrine organ in the body (Rehfeld, 1998; Breer *et al*., 2012). Thus, a letter system was devised to classify distinct EECs types according to their produced hormones or ultrastructural characteristics detected using electron microscopy. EECs can also be divided into two types depending on their morphological characteristics: closed-type and open-type cells.

Glucagon-like peptide (GLP) is a key intestinal hormone that exists in two forms: GLP-1 and GLP-2. These two hormones contribute significantly to nutritional homeostasis by controlling food intake, digestion, absorption, and a variety of physiological responses in the gut (Dubé and Brubaker, 2004). GLP-1 is involved in the proliferation of pancreatic β cells, the delaying of gastric emptying, the reduction of intestinal motility, and the promotion of the growth of the intestine (Tolessa *et al*., 1998; Nauck, 1998; Drucker, 2006; Kissow *et al*., 2012). GLP-2, on the other hand, is widely recognized for its intestinal trophic effects. It promotes epithelial cell proliferation, inhibits epithelial cells apoptosis, lengthens microvilli, decreases gastric motility, and modulates hexose transport (Cheeseman and Tsang, 1996; Wojdemann *et al*., 1999;

Benjamin *et al*., 2000; Brubaker, 2006; Guan *et al*., 2006). The physiological effects of GLP-1 and GLP-2 are inextricably linked since both peptides are synthesized from the same proglucagon precursor and released by the same intestinal endocrine L-cells. A recent research demonstrated that GLP-1 is colocalized with NT in the same EECs in the small intestine of chicken (Nishimura *et al*., 2017). NT has been shown to have a variety of physiological activities, including regulating gastric and intestinal motility, inducing pancreatic and biliary secretion, enhancing mucosal development and inflammation response, and increasing vascular permeability (Harper *et al*., 1984; Thor and Rosell, 1986; Gui *et al*., 2001; Evers, 2006; Gross and Pothoulakis, 2007).

However, an ultrastructural study demonstrated that L cells in the chicken ileum had a long cytoplasmic process and were covered with microvilli on the apical surface (Nishimura *et al*., 2013). Another research discovered that intestinal N cells in the ileum of chickens have a long cytoplasmic process furnished with microvilli that reaches to the intestinal lumen (Sundler *et al*., 1982). EECs with this architecture is referred to as open-type cells, and they produce a variety of peptide hormones (Hiramatsu, 2020). These open-type EECs are considered primary chemosensory agents for assessing the nutrients in the intestinal lumen (Breer *et al*., 2012), and by monitoring the lumen contents, they secrete their hormones through endocrine and paracrine processes (Gribble and Reimann, 2016). Furthermore, the microvilli of open-type cells are employed as receptor sites for various receptors and to establish a signal transaction with the intestinal lumen. Numerous studies have shown that individual nutrients may have an influence on the synthesis and release of hormones from their respective cells, which are necessary for the execution of different physiological and biological processes in the gastrointestinal system.

Taking into consideration the above evidence, the primary hypothese of this dissertation is that dietary nutrients influence the structure and function of the mucosa of the chicken small intestine. The objective of this dissertation was comprehensively examined in the chicken ileum utilizing a variety of methods such as morphometry, immunohistochemistry, and *in situ* hybridization, amongst others. Before this work, there was very little evidence on the effects of dietary nutrients on the alteration of mucosa and functional diversity in the chicken ileum.

The first experiment was conducted to evaluate the effects of dietary CHO level on the enterocyte microvilli in the chicken ileum. The microvilli in the higher CHO groups were straight and densely packed. In the lower CHO groups, microvilli fragmentation and huge spacing between neighbors microvilli were found. The length, width, and linear density of microvilli were all significantly higher in the higher CHO groups than in the other groups. The fluorescence intensities of microvillar proteins villin and β-actin were higher in the higher CHO groups. The frequency of GLP-2-immunoreactive cells and cells expressing PG mRNA showed an increasing trend with increasing the level of CHO in the diets. These findings, therefore, clearly indicate that dietary CHO is the most crucial nutrient for the maintenance of enterocyte microvilli in the chicken ileum, which is reliant on GLP-2 release by intestinal L cells. The second experiment was designed to determine the influence of dietary protein level on NT-immunoreactive cells. Most of NT-immunoreactive cells in the villus epithelium showed pyramidal or spindle-like shape with a long cytoplasmic process reaching the intestinal lumen in the control group, but cells in a round or oval shape were found in lower CP groups, particularly in 0% CP group. Interstingly, the frequency of NT-immunoreactive cells was significantly higher in the groups with higher level CP. A statistically significant relationship was also observed

between the frequency of occurrence of NT-immunoreactive cells and the amount of protein intake on a daily basis. Following these findings, it is probable that the ingested protein acts as a potential signal to NT synthesis and release by N cells in the chicken ileum. Moreover, in the third experiment, double immunofluorescence staining demonstrated that GLP-1 is colocalized with NT and demonstrated that the effects of dietary CHO on these type cells. These colocalized cells showed a long cytoplasmic process reaching the intestinal lumen in the control group. On the other hand, round or oval shape cells were predominant in the CHO-free group. These morphological differences unequivocally demonstrate that endocrine cells expressing both GLP-1 and NT in the higher CHO groups are capable of detecting the presence of CHO in the lumen. In addition, the ratio of the endocrine cells expressing both GLP-1 and NT was significantly higher in the control group and decreased with declining the CHO level in the diets. Cells expressing PG mRNA and NTP mRNA were apparently higher in the control group. These findings suggest that dietary CHO level is a potent stimulator to increase the occurrence of the endocrine cells expressing both GLP-1 and NT in the distal ileum of chicken. Taking these observations together, it is hypothesized that particular dietary nutrients may stimulate the functional capacity of the chicken ileum.

A recommendation based on my findings in this dissertation is to contemplate the idea that specific dietary nutrients may have an impact on morphological and functional alterations of the intestinal mucosa in chickens. To the best of my knowledge, no previous research has been conducted on the impact of dietary nutrients on the mucosa formation and function of chicken intestine prior to this study. Considering that the dietary ingredient had an enormous effect on the morphological features of the intestinal mucosa, and these results might be used an indicator to study further in connection to nutrition to

develop feed formulations that promote optimal digestion and absorption in order to increase chicken production. These structural alterations in the mucosa would also serve as an indicator for nutritionists in the chicken industry to utilize nutrients in the most effective manner possible. Furthermore, variations in the frequency of endocrine cells observed in the current study reflect changes in the functionality of the gut. It is believed that GLP-1 and NT are involved in the control of gut motility, which has an impact on the digestion and absorption of nutrients. In this dissertation, the frequency of cells colocalized GLP-1 and NT was increased by dietary nutrients that induced functional alterations in the chicken intestine. These results may assist personnel involved in poultry industry in enhancing their chicken intestine functioning capacity. Thus, the current findings imply that there is possibility to modify the mucosa functionality by manipulating specific nutrients. As a consequence, these findings may shed fresh light on how to influence mucosal activities by specific dietary nutrients. Therefore, the present dissertation study contributed to the understanding of the effects of specific dietary nutrients on the structural variety and functional capacity of the mucosa, which may be used as primary evidence for systematic research further and to increase the performance of a diverse group of avian species.

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