PROTEOMIC PROFILE INVOLVED WITH CARNOSINE AND CARNOSINE DERIVATIVE SYNTHESIS OF KORAT CHICKEN

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โปรติโอมิกส์โปรไฟล์ที่เกี่ยวข้องกับการสังเคราะห์สารคาร์โนซีนและอนุพันธ์ของสารคาร์โนซีนในไก่โคราช

นางสาวชนัดดา สุวรรณวิชนีย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
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ชนัดดา สุวรรณวิชนีย์: โปรติโอมิกส์โปรไฟล์ที่เกี่ยวข้องกับการสังเคราะห์สารคาร์โนซีนและอนุพันธ์ของสารคาร์โนซีนในไก่โคราช (PROTEOMIC PROFILE INVOLVED WITH CARNOSINE AND CARNOSINE DERIVATIVE SYNTHESIS OF KORAT CHICKEN) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. อมรรัตน์ โมพี, 96 หน้า.

คำสำคัญ: คาร์โนซีน/β-Alanine/L-Histidine/molecular pathway/โปรติโอมิกส์โปรไฟล์/ไก่กลุ่มโตช้า

วัตถุประสงค์ของการศึกษานี้คือ ศึกษากลไกทั้งหมดที่เกี่ยวข้องกับการสังเคราะห์คาร์โนซีนและผลที่เกิดขึ้นจากการสังเคราะห์คาร์โนซีนในไก่โคราช (KRC) โดยการใช้โปรติโอมิกส์เทคนิค ดังนั้นในการศึกษานี้จึงได้กระตุ้นการสังเคราะห์คาร์โนซีนในเนื้อไก่ด้วยการเสริมกรดอะมิโนที่เป็นสารตั้งต้นของการสังเคราะห์คาร์โนซีนในอาหารเพื่อให้เนื้อไก่มีการสังเคราะห์คาร์โนซีนที่แตกต่างกัน โดยสุ่มไก่โคราชเพศเมียอายุ 3 สัปดาห์ จำนวน 400 ตัว แบ่งเป็น 4 กลุ่มปัจจัยทดลอง (กลุ่มละ 5 ซ้ำ/ซ้ำละ 20 ตัว) ได้แก่กลุ่มควบคุม, กลุ่มที่เสริม 0.5% L-histidine, 1% β-alanine และ 0.5% L-histidine+ 1% β-alanine ทำการเก็บข้อมูลประสิทธิภาพการเจริญเติบโตที่ช่วงอายุ 3-6 สัปดาห์ และ 6-10 สัปดาห์ เมื่อไก่อายุ 10 สัปดาห์ ไก่โคราช 5 ตัวต่อซ้ำ ถูกฆ่า และเก็บเนื้ออกเพื่อทำการวิเคราะห์ลักษณะคุณภาพเนื้อ ในขณะเดียวกันไก่โคราช 2 ตัวต่อซ้ำ ถูกฆ่า และเก็บเนื้ออกเพื่อทำการวิเคราะห์ปริมาณคาร์โนซีน, แอนเซอรีน, thiobarbituric acid reactive substances (TBARS), องค์ประกอบทางเคมีในเนื้อ และโปรติโอมิกส์โปรไฟล์

ผลการศึกษาพบว่าไก่โคราชสามารถสังเคราะห์สารคาร์โนซีนเพิ่มขึ้นได้ เมื่อถูกกระตุ้นด้วยกรดอะมิโนที่เป็นสารตั้งต้น และเพิ่มได้สูงที่สุดในกลุ่มที่เสริมด้วย L-histidine และ β-alanine ร่วมกัน ($P<0.05$) แต่ไม่ส่งผลต่อการเพิ่มขึ้นของปริมาณแอนเซอรีน ($P>0.05$) นอกจากนี้การเพิ่มขึ้นของคาร์โนซีนไม่ส่งผลต่อประสิทธิภาพการเจริญเติบโต ($P>0.05$) ในขณะที่การวิเคราะห์องค์ประกอบหลัก (principal component analysis) แสดงให้เห็นว่าการเพิ่มขึ้นของคาร์โนซีนมีความสัมพันธ์กับการชะลอการลดลงของ pH_{45} นาที, การเพิ่มขึ้นของ α-helix และการลดลงของ drip loss, cooking loss, shear force, TBARS และ β-sheet แต่อย่างไรก็ตามการเสริมกรดอะมิโนตัวใดตัวหนึ่งอาจส่งผลกระทบเชิงลบต่อลักษณะเนื้อสัตว์ เนื่องจากมีความสัมพันธ์กับการเพิ่มขึ้นของ β-sheets, β-turns, และ aliphatic bending groups และการลดลงของ α-helix

ในการศึกษาโปรติโอมิกส์โปรไฟล์ตัวอย่างเนื้อไก่ในกลุ่มการทดลองที่เสริมด้วย β-alanine+L-histidine ถูกเลือกเพื่อเป็นตัวแทนของกลุ่มคาร์โนซีนสูงเปรียบเทียบกับเนื้อไก่จากกลุ่มควบคุมที่เป็นตัวแทนของกลุ่มคาร์โนซีนต่ำ ผลการศึกษาพบว่าในจำนวน 152 โปรตีนที่ระบุอยู่ใน common protein มี 6 โปรตีน ได้แก่โปรตีน HSPA8, MYOM2, FABP3, LUM, H4-VIII และ HSPA2 ที่มีการแสดงออกสูงในกลุ่ม high carnosine (HC) และ โปรตีน 2 ชนิด ได้แก่ โปรตีน Titin isoform Ch12

และ Connectin ที่แสดงออกสูงในกลุ่ม Low carnosine (LC) ($P<0.05$) จากการวิเคราะห์ function enrichment, Kyoto Encyclopedia of Genes และ Genomes pathway และ protein–protein network พบว่า HSPA2, HSPA8, PDIA6 และ ERP29 ซึ่งเป็นโปรตีนที่เกี่ยวข้องกับกระบวนการ Protein processing ใน endoplasmic reticulum pathway มีความสัมพันธ์กับการสังเคราะห์คาร์โนซีนใน Pectoralis major muscle ของไก่โคราช นอกจากนี้การเพิ่มขึ้นของคาร์โนซีนมีความสัมพันธ์กับการกระตุ้นการแสดงออกของโปรตีน Titin isoform Ch12 (TTN), Connectin, และ M-protein (MYOM2) เพื่อควบคุมสมดุลภายในเซลล์ และความเครียดในระหว่างกระบวนการหดตัวของกล้ามเนื้อ รวมถึงกล้ามเนื้อหัวใจ นำไปสู่การปรับปรุงการทำงานของกล้ามเนื้อและสุขภาพของสัตว์ ซึ่งสอดคล้องกับผลที่ได้จากการวิเคราะห์องค์ประกอบหลัก (PCA) พบว่าคาร์โนซีนมีความสัมพันธ์ในทิศทางบวกกับ Lumican, Fatty acid binding protein 3, HSPA8, HSPA2, MYOM2, β-turn, α-helix, และ pH45 min, และมีความสัมพันธ์เชิงลบกับ TTN, Connectin, β-sheet, drip loss, cooking loss, shear force, และ TBARS

ผลการศึกษานี้สามารถสรุปได้ว่าการสังเคราะห์คาร์โนซีนที่สูงขึ้นอาจเกิดขึ้นเมื่อเซลล์ต้องการที่จะคืนสภาพเข้าสู่สภาวะสมดุล ซึ่งส่งผลให้การทำงานของกล้ามเนื้อดีขึ้น นำไปสู่การไม่มีผลเสียต่อสุขภาพหรือลักษณะของเนื้อสัตว์ ดังนั้น การค้นพบนี้จึงสร้างความมั่นใจในการก้าวไปสู่การปรับปรุงพันธุกรรมเพื่อเพิ่มความสามารถในการสังเคราะห์คาร์โนซีนโดยไม่ส่งผลเสียต่อการทำงานระดับโมเลกุลในไก่

สาขาวิชาเทคโนโลยีและนวัตกรรมทางสัตว์
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ลายมือชื่อนักศึกษา ชนิดดา สุวรรณวิชชุนี่ย์
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CHANADDA SUWANVICHANEE : PROTEOMIC PROFILE INVOLVED WITH CARNOSINE AND CARNOSINE DERIVATIVE SYNTHESIS OF KORAT CHICKEN. THESIS ADVISOR : ASSOC. PROF. AMONRAT MOLEE, Ph. D., 96 PP.

Keyword: CARNOSINE/β-ALANINE/L-HISTIDINE/MOLECULAR PATHWAY/PROTEOMIC APPROACH/SLOW-GROWING CHICKEN

The objective of this study was to study the global mechanism and consequently related to carnosine synthesis using a proteomic approach in Korat chickens (KRCs). Thus, in this study, amino acids which are substrates of carnosine synthesis were used to activate different carnosine syntheses in the chicken meat. The 3-week-old female KRC were used in this study, and they were randomly allocated to 4 different diets: control (5 replicates per group/20 chickens per replicate), dietary supplementations 0.5% L-histidine, 1% β-alanine and 0.5% L-histidine+1% β-alanine. Each treatment included 5 replicates of 20 chicken per pen. The growth performances were measured at 3-6 weeks and 6-10 weeks of ages. At the 10 weeks of age, 5 chickens per replicate were slaughtered and breast meat were collected to measure meat quality. At the same time, 2 chickens per replicate were slaughtered and breast meat were collected to measure carnosine content, anserine content, thiobarbituric acid reactive substances (TBARS), biochemichal compound and proteomic profile.

The results showed that KRCs chickens synthesized more carnosine when they were supplemented with the substrates of carnosine synthesis and the highest carnosine content existed in the group which were supplemented with L-histidine and β-alanine together ($P<0.05$), but these did not have any effect on anserine synthesis ($P>0.05$). Moreover, an increase of carnosine showed no significant difference in growth performance ($P>0.05$). Principal component analysis (PCA) analysis revealed that the increase of carnosine was related to a gradual decline in $pH_{45\ min}$ with an increase in α-helix and decreasing drip loss, cooking loss, shear force, TBARS, and β-sheet. However, supplementation with only β-alanine or L-histidine was related to increased content of β-sheets, β-turns, and aliphatic bending groups

and decreased content of α-helix groups, which may negatively affect meat charesterictics.

Regarding the study of proteomics profiles, the meat in the group which was supplemented with β-alanine+L-histidine was selected to represent the high-carnosine content group compared with a control group that represented the low-carnosine content group. The result found that among 152 proteins listed in the common proteins, 6 proteins namely HSPA8, MYOM2, FABP3, LUM, H4-VIII, and HSPA2, were highly expressed in the high carnosine group (HC) and 2 proteins namely titin isoform Ch12 and connectin, were highly expressed in low carnosine (LC) group ($P<0.05$). From function enrichment, Kyoto Encyclopedia of Genes and Genomes pathway and protein–protein network analyses found that HSPA2, HSPA8, PDIA6, and ERP29 which was enriched in protein processing in the endoplasmic reticulum pathway are associated with carnosine synthesis in Pectoralis major muscle of the slow-growing KRC. Moreover, carnosine is mainly involved in stimulating Titin isoform Ch12 (TTN), Connectin, and M-protein (MYOM2) expressions for maintaing homeostasis and to regulate stress in skeletal and cardiac muscle cells that finally resulted in the desired meat characteristics and health. This result is consistent with the PCA result which found that carnosine content was positively loading correlated with LUM, FABP3, HSPA8, HSPA2, MYOM2, β-turn, α-helix, and $pH_{45\ min}$, and negative loading correlation with TTN, Connectin, β-sheet, drip loss, cooking loss, shear force, and TBARS.

From this study it can be concluded that higher carnosine synthesis may occur when cells need to recover homeostasis, leading to improved muscle function with no adverse effects on health or meat characteristics. Thus, these findings provide the confidence to step forward for genetic improvement to enhance the ability of carnosine synthesis without adverse impact on the molecular function in chickens.

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Chanadda Suwanvichanee

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LIST OF ABBREVIATIONS

KRC	=	Korat chicken
%	=	Percent sign
wk	=	Week
kg	=	Kilogram
g	=	Gram
℃	=	Degree Celsius
H	=	Hour
cm^3	=	Cubic centimeter
mm	=	Millimeter
s	=	Second
w/v	=	Weight/Volume
mo	=	Month
g	=	Relative centrifugal fields
min	=	Minute
ml	=	Milliliter
M	=	Molar
nm	=	Nanometer
µm	=	Micrometer
mm	=	Millimeter
HPLC	=	High performance chromatography
mM	=	Millimolar
cm^{-1}	=	The number of waves per centimeter
µg	=	Microgram
µl	=	Microliter
mg	=	Milligram
m/z	=	Mass-to-charge ratio

CHAPTER I
INTRODUCTION

1.1 Introduction

Korat chickens (**KRCs**) is a crossbred chicken between Thai indigenous Leung Hang Khao sires, and Suranaree University of Technology dams. The aimed of KRCs development is recognized as a tool in the occupation of small-scale farmers in the country which leads to a Sustainable Development Goals to achieve zero hunger, food security, nutrition improvement and to promote sustainable agriculture (UN DESA, 2018). Currently, the development of KRCs is moving to the second S-CURVE - aiming for value-added chicken as the source of natural extract to produce dietary supplement, cosmetical products and medicine. Consequently, the ultimate goal of this study is to develop Korat chicken to be the source of carnosine and its derivative.

According to global consumer trends seem to have a paying attention to nutrient-enriched foods for certain health conditions such as diabetes, obesity, and cardiovascular diseases (Pinto et al., 2021). This result has led to the trend in the future that the consumers will consume food that can respond to healthy, tasty and inexpensive aspects. Carnosine has beneficial effects on human health (Hisatsune et al., 2016; Xing et al., 2021) since it can demonstrate the ability of the suspension of biochemical processes via scavenging free radicals, chelating metals, and maintaining buffer capacity (Güner and Alpsoy. 2015). Of interest, carnosine is mainly found in muscle and brain tissue of vertebrates or mammals (Everaert et al., 2011), and from previous research found that slow-growing chicken meat consisted of high amount of carnosine than fast-growing chicken (Tian et al., 2007, Barbaresi et al., 2019). Moreover, Mateescu et al. (2012) and D'Astous-Pagé et al. (2017) had reported that genetic effect is one of varieties source of carnosine content in animal. As the mentioned, thus genetic improvement makes KRC can synthesize more carnosine, and can be a source of carnosine will be our breeding goals.

The mechanism of carnosine synthesis in animals was first investigated in mice by Everaert et al. (2013). Carnosine is remarkably abundant in muscles and the brain, which it is synthesized by carnosine synthase using β-alanine and L-histidine as substrates (Harris et al., 2012). However, previous knowledge related with carnosine synthesis in molecular level of chicken were generated by Cong et al. (2017); Qi et al. (2018); Qi et al. (2021). They studied in fast-growing chickens and found carnosine synthesis was related to expression of genes histidine decarboxylase (***HDC***), proton-coupled oligopeptide transporters (***PEPT1***), solute carrier family 6 member 6 (***SLC6A6***) and carnosine synthase (***CARNS***). In addition, Khumpeerawat et al. (2021) and Sharma et al. (2022), studied in slow-growing chickens and found that genes carnosine synthase1 (***CARNS1***) and solute carrier family 36, member 1 (***SLC36A1***) transporter were involved in carnosine synthesis. Moreover, Drozak et al. (2010) reported that ATP-grasp domain-containing protein 1 (**ATPGD1**) was related to carnosine synthesis. All of them are specific genes, and protein were related to carnosine synthesis, global pathway, genes or proteins involved with carnosine synthesis, carnosine function, and consequences of the function, however, are still questionable.

However, the development of the chicken with higher carnosine needs to know all the consequences of chicken, whether with performance, meat characteristic, or chicken health. Unfortunately, in chickens, the study about carnosine synthesis in molecular level is limited. Although some studies reported genes involved in carnosine synthesis, they cannot be directly correlated to actual protein abundance (Vogel et al., 2010). Proteins and their enzyme entities determine the phenotypic diversity arising from a set of common genes. Furthermore, posttranslational modifications revealed by proteomics regulate structure, function, localization, maturation, and turnover of proteins (Rifai et al., 2006). Therefore, information about proteomics will be illustrated underlying the interaction between genes and the environment, and its global mechanisms related to carnosine synthesis.

Therefore, information about proteomics is illustrated underlying the interaction between genes and the environment, and its related mechanisms for confidence to take a step toward genetic improvement to enhance the ability of carnosine synthesis without adversely impacting the molecular function in chickens.

The objectives of this study are to find the global mechanism of carnosine synthesis and consequently related to carnosine synthesis in Korat chicken using a proteomic approach. In this study, we designed the difference in carnosine content by supplementation substrates of carnosine synthesis (β-alanine and L-histidine) in the diet for activating carnosine synthesis in KRC meat. Hence, based on the highest carnosine content result, we investigated further about the proteomic profile and pathway related to the increase of carnosine synthesis and meat quality trait in KRC meat. The knowledge of this study will give an understanding the related pathway of carnosine synthesis leading to the precise find genes marker, and provide confidence to take a step toward genetic improvement to enhance the ability of carnosine synthesis without adversely impacting the molecular function in chickens.

1.2 Research objectives

The objectives of this study were:

1. To study the differences of carnosine content impacting on meat characteristics in Korat chicken.

2. To study the differences of proteins expression related to carnosine synthesis in Korat chicken.

1.3 Research hypotheses

1. Found the influence of the differences of carnosine content on meat characteristics in Korat chicken.

2. Found the differences of proteins expression related to carnosine synthesis in Korat chicken.

1.4 Scope of the study

The objective of this study is to find the global mechanism of carnosine synthesis and consequently related to carnosine synthesis in Korat chicken using a proteomic approach. The 3-week-old female KRC were used in this study, and they were randomly allocated to 4 different diets: control, dietary supplementations 0.5% L-histidine, 1% β-alanine and 0.5% L-histidine+1% β-alanine. Each treatment

included 5 replicates of 20 chicken per pen. The growth performances were measured at 3-6 weeks and 6-10 weeks of ages. At the 10 weeks of age, 5 chickens per replicate were slaughtered and breast meat were collected to measure meat quality. At the same time, 2 chickens per replicate were slaughtered and breast meat were collected to measure carnosine content, anserine content, TBARS, biochemicals compound and proteomic profile.

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CHAPTER II

LITERATURE REVIEWS

2.1 Korat chicken (KRC)

Korat chicken is generated from Thai indigenous chicken (Sire) and SUT synthetic breed (Dam). The goal of Korat chicken development is to get a sustainable carrier for small- to moderate-sized holders of Thai farmers. Korat chicken is categorized as a slow-growing chicken (Poompramun et al., 2021) because its growth rate is around 24 g/day, and its body weight reaches market weight (approximately 1.2 to 1.3 kg) within 9 to 10 weeks of age (Hang et al., 2018). The meat characteristics of Korat chicken are low fat, high protein and good quality (Katemala et al., 2021) resulting to a responsible on meat quality, safety, and animal welfare (Skaperda et al., 2019). However, the lower performance of KRC, particularly of female KRC, is a significant disadvantage. Therefore, adding value of female KRC by genetic improvement to enrich some biochemical compounds in meat that are beneficial for consumer health is one strategy to improve the competitiveness of smallholder farmers and differentiate KRC from commercial broilers.

2.2 The consumer trends in food demand

Thailand is going to be aged society, which means 10% of the total population has equal or over of 60 years of age; and it will completely enter elderly society in 2021 (The Situation of Thai Elderly, 2014) as shown in Figure 2.1. Moreover, in the future, global consumer trends seem to have on paying attention to nutrient-enriched foods for certain health conditions (Pinto et al., 2021). It is revealed that healthy food trending is still going up after the COVID 19 pandemic. The consumer behavior changes and concern about a fatal health risk resulting in the demand for consumption of health improvement are increased (Janssen et al., 2021). The changes will affect demand of consumers including foodstuffs, wares, and residence whereas elderly has reduced demand for food due to physical and mental changes.

As a result, healthy food from nature has become more popular. Therefore, this is an opportunity to add product value and steal market share by adding bioactive compounds in food products.

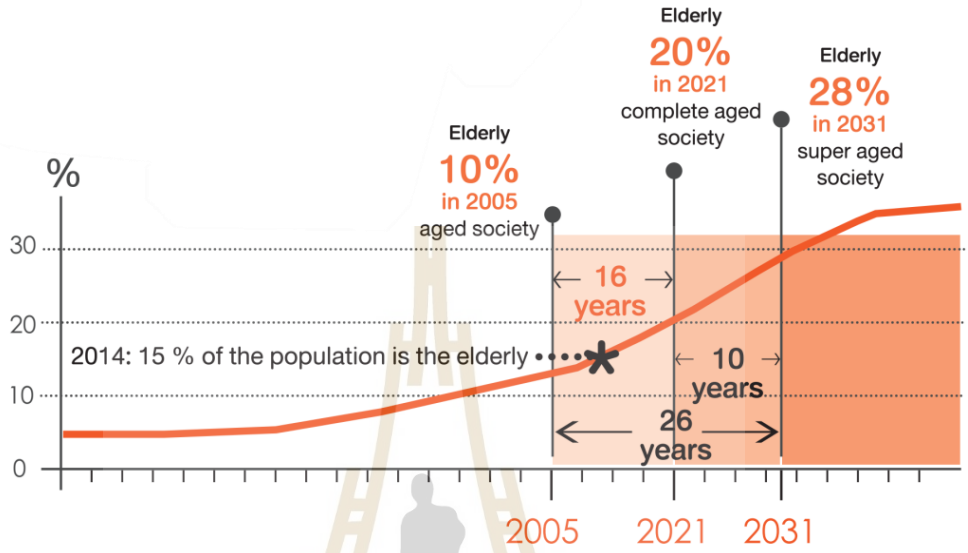

Figure 2.1 The Thai Population is aging rapidly.
Source: The Situation of Thai Elderly (2014).

2.3 Carnosine and its derivatives

Carnosine was first discovered by the Russian biochemist Vladimir Gulevich in 1900 as he searched for an unknown nitrogen compound from meat extracts. Therefore, he named the compounds discovered: “carnosine” (cani is Latin, meaning meat). Carnosine is a dipeptide, consisting of a naturally occurring histidine compound. The structure of carnosine and its derivatives are shown in Figure 2.2. The binding between β-alanine and L-histidine has a specific enzyme “carnosine synthase” which are found in muscle and brain. Carnosine will be degraded by specific enzymes “carnosinase” which is found in liver, kidney, blood, etc. (Begum et al., 2005), so carnosine will not break down by enzyme dipeptidase (Boldyrev et al., 2013). Carnosinase is found in most tissues except muscle structure. That is why the amount of carnosine in the musculoskeletal structure is high (Culbertson et al., 2010).

β-alanine is non-essential amino acid and a non-proteinogenic amino acid that the body can synthesize from the liver. β-alanine can be synthesized from many

pathways such as aspartate amino acids, the breakdown of uracil, α-alanine forms, acetyl-CoA or oxaloacetic acid etc. (Wang et al., 2014); while histidine is essential amino acid requirements of the animal and must be obtained from food. This structure is composed of the imidazole ring at pKa of 6.0. It is possible that histidine residue will be combined with β-alanine to increase the buffering properties (carnosine the pKa=6.72). Another reason is that histidine residues will cause imbalances and toxicity; as a result, histidine binds to beta-alanine. However, β-alanine amino acids are limited to synthesize carnosine. So, it is necessary to get from food as well (Brosnan and Brosnan. 2020).

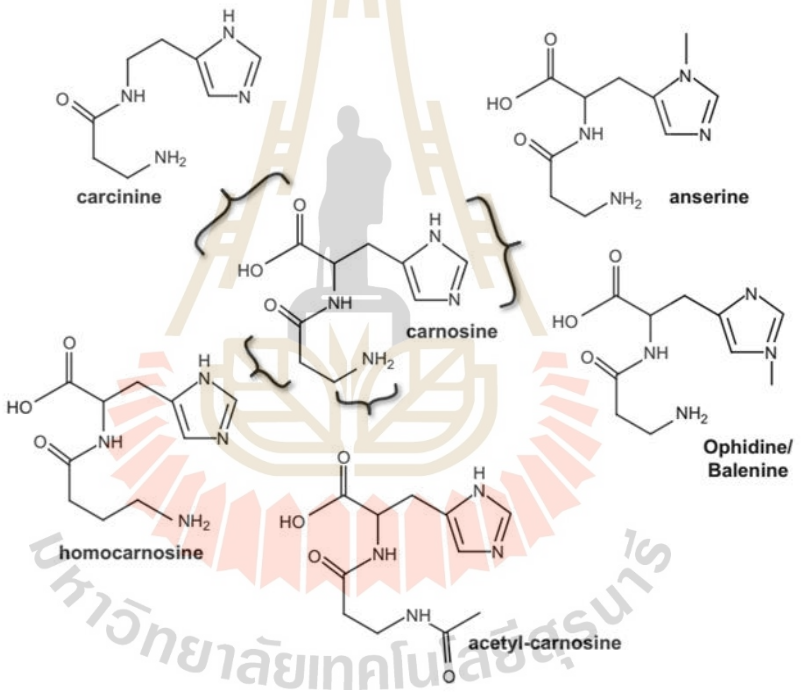

Figure 2.2 Structure of carnosine and its derivatives.

2.4 The sources of carnosine and its derivatives

Carnosine is mainly found in muscle and brain tissue of vertebrates or mammals. It is rarely found in other organs, plants (including vegetarian) and invertebrates (Derave et al., 2010). The natural derivatives of carnosine are anserine and ophidine, since the presence of methyl compounds in the histidine molecule called histidine-containing dipeptides (Boldyrev et al., 2013). Other carnosine

derivatives are composed of homocarnosine (histidine and γ-aminobutyrate) and acetyl-carnosine (Culbertson et al., 2010), which occurred by carnosine metabolism shown in Figure 2.3.

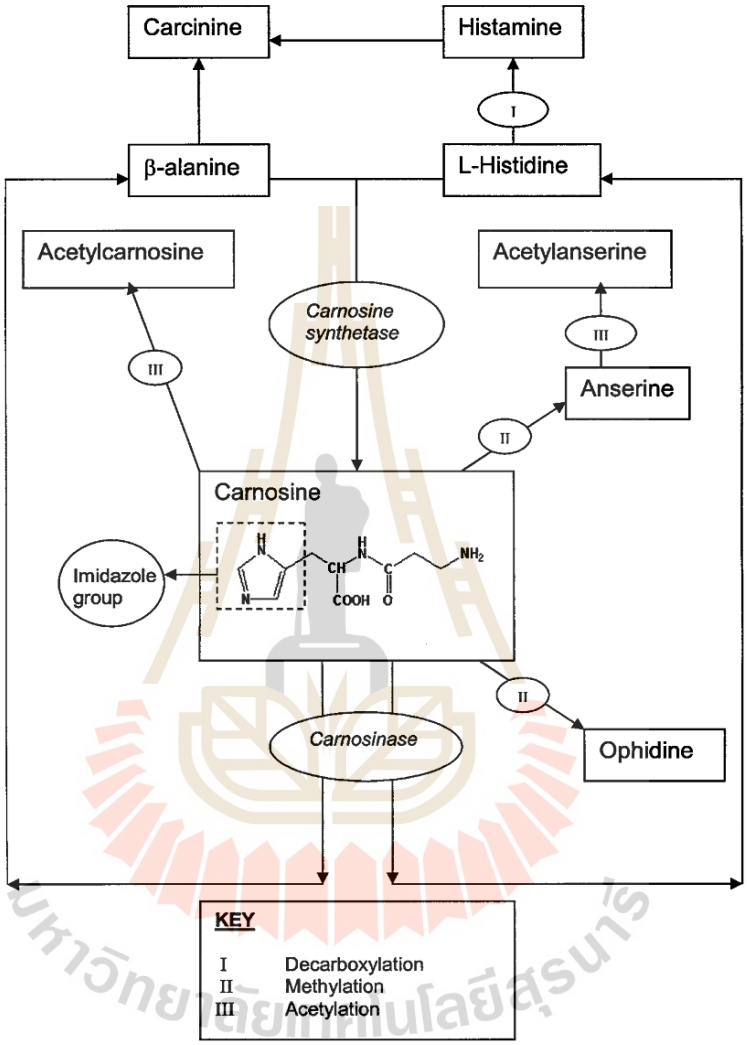

Figure 2.3 The pathway of carnosine and its derivatives.
Source: Culbertson et al. (2010).

The natural carnosine derivatives in animals that have been reported are composed of carnosine anserine, ophidine, balenine, and homocarnosine as showed in Table 2.1. Carnosine and its derivatives are accumulated in muscle in many kinds of animals; moreover, the difference in muscle types is one factor of the carnosine content. Anserine is more in poultry than mammals and it has the same role as

carnosine. Therefore, chicken meat is an important source of carnosine and its derivatives.

Table 2.1 The amount of carnosine anserine, balenine and homocarnosine in animals and other vertebrates.

Bree	Musc	Carnosine	Anserine	Balenine	Homocarnosine	Unit	Reference
Chick	Breast	149.27±7.06	675.59±33.32	5.50±0.61	-	(mg/ 100 g)	(Mora et al., 2007)
Pork	Loin	462.33±16.86	10.76±1.23	33.40±1.88	-		
Chick	Breast	180.0±10.9	772.2±50.3	4.61±0.56	-	(mg/	(Aristoy and
	Leg	63.0±5.8	233.7±18.7	10.1±1.1	-	100 g)	Toldra.,
	Blend	109.8±10.2	489.3±42.2	4.14±0.31	-		2004)
Hors	Fillet	1.73±0.083	0.010±0.0026	-	0.0037±0.000	%w/w	(Peiretti et
Pork	Loin	1.76±0.015	0.050±0.0031	-	0.18±0.020		al., 2011)
Beef	Fillet	1.43±0.18	0.16±0.015	-	0.014±0.004		
Rabb	Hindl	0.39±0.015	1.12±0.12	-	0.027±0.002		
Chick	Breas	0.76±0.25	1.46±0.43	-	0.11±0.059		
Turky	Breas	0.62±0.15	2.19±0.78	-	0.19±0.062		
Beef	Leg	26.1±3.7	5.94±1.75	0.103±0.03	-	µmol/ g wet t.	(Abe and Okuma, 1995)
Hors	Leg	42.6±12.6	0.176±0.030	0.019±0.00	-		
Chick	Leg	5.7±1.7	17.1±3.7	0.055±0.03	-		
	Breas	10.4±1.3	32.0±1.4	0.197±0.03	-		
Turky	Leg	4.53±0.68	20.5±1.9	0.077±0.01	-		
	Breas	11.2±1.3	46.0±0.8	0.810±0.02	-		

2.5 The role of carnosine on human health

Carnosine has a role by intermediary to protect advanced glycoxidation end-products (**AGEs**) and advanced lipoxidation end-products (**ALEs**) as shown in Figure 2.4. Glucose, other reducing sugars, or polyol pathway that glucose is converted to sorbitol with NADPH as a cofactor, and sorbitol is converted to fructose. These sugars,

when interacting with an amine of amino acid, lead to aldimine unstable and change into Shiff bases form. Then, these compounds will be rearranged into stable early glycation products that are called Amadori products Intermediate stage. This can occur through every step of the glycation process. Glycation is the first step of AGEs that is the browning process by enzymatic-free. AGEs are the product of the carbonyl group of reducing sugars with the amino groups of lysine such as dehydration oxidation, Maillard reaction, polyol pathway, catabolism of ketone body, or treonine. Lipid peroxidation leads to Amadori product breakdown into groups of reactive dicarbonyl compounds such as glyoxal, methylglyoxal and deoxyglucose (Hipkiss et al., 2013). As a result, these compounds are sensitive to bind to free amino acids and are classified as an intermediate product of the AGEs and ALEs process.

Carnosine acts as an inhibitor of AGEs through different processes as follows: 1. Inhibit protein from Schiff base due to the effect of Transglycating (dissolving AGE cross-ling back into sugars with proteins); 2. Inhibit the oxidation of sugars to be free radicals, Reactive carbonyl species (**RCS**) (carbonyl free radicals); 3. Drive free radicals from the body; 4. Inhibit the oxidation of Amadori Product to AGEs. All processes are shown in Figure 2.4.

Moreover, carnosine can inhibit lipid oxidation end products of proteins that occur under RCS radicals with a mechanism: 1. Inhibit lipid oxidation to RCS from the body; 2. Expel free RCS radicals from the body; and 3. Inhibit the destruction of protein in a way that carnosine will react (Not related to enzymes) with the carbonyl group of proteins, a process known as 'carnosinylation' (Boldyrev et al., 2013).

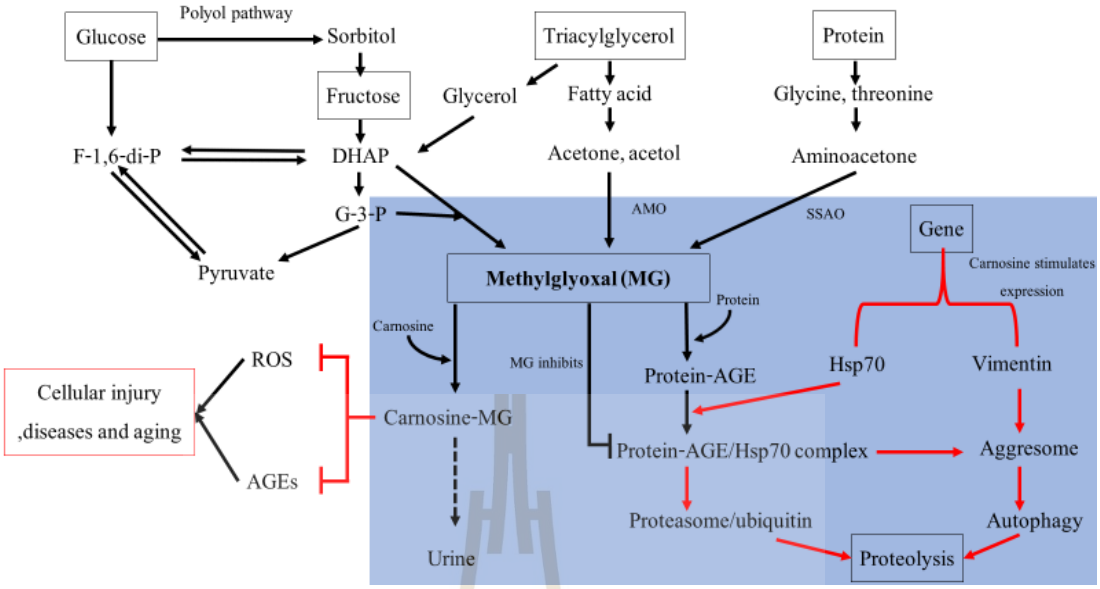

Figure 2.4 Metabolic sources of methylglyoxal (MG) and the possible role of carnosine in scavenging MG, suppressing the formation and catabolism of abnormal proteins (shown as an example) of protein-AGEs. Protein-AGEs cause inflammation and ageing. MG-carnosine is excreted in urine.

Source: adapted from Hipkiss et al. (2013).

The toxin from glycation is advanced lipoxidation end-products and advanced glycoxidation end-products, both known as glycotoxin, which play an important role in the pathogenesis of humans. A high glycotoxin was detected in patients of Diabetes, Kidney disease, Heart disease, Cancer, and Degenerative neurosis. The binding between glycotoxin and a hemoglobin in the blood stops its work, due to blood cells being unable to carry oxygen to various cells in the body. Carnosine in the mitigation of various diseases is that carnosine inhibits ALEs and AGEs (Figure 2.4) (Hipkiss et al., 2013). Its role in reducing diabetes: Carnosine treatments (100 and 200 mg/kg) reduce hyperglycemia, normalize blood lipid levels, and reduce liver damage (Soliman et al., 2007, Lee et al., 2005, Nagai et al., 2003). Moreover, Carnosine can prevent and help treatment of Alzheimer's disease. According to research papers by Hisatsune et al. (2016); Rokicki et al. (2015), carnosine may be able to maintain cognitive function, including verbal episodic memory impairment in the elderly. Regarding anti-aging: when carnosine was used as a dietary supplement in rats,

naturally occurring carnosine was found to have anti-aging effects as well as to significantly improve the animal's endogenous physiology (Boldyrev et al., 1999 and Gallant et al., 2000). Moreover, carnosine increases life expectancy and quality of life by reducing lipid peroxides production and reducing the effects of free radicals on protein membranes (Yuneva et al.,2002). In addition, carnosine is currently used as a compound in anti-cancer drugs. From previous test, it was found that carnosine showed anti-tumor effect in rats, especially Glioblastoma brain tumors (Gaunitz and Hipkiss., 2012, Holliday and McFarland, 1996) (shown in Table 2.2). This evidence is indicated that carnosine is an important role in human health.

Table 2.2 The role of carnosine on human health.

Disease	Animal	Reference
Antiaging	Rat	Boldyrev et al. (1999)
		Gallant et al. (2000)
		Yuneva et al. (2002)
Diabetes	Rat	Soliman et al. (2007)
		Lee et al. (2005)
		Nagai et al. (2003)
Alzheimer's	Humans' elderly	Hisatsune et al. (2016)
		Rokicki et al. (2015)
Cancer	Rat	Gaunitz and Hipkiss. (2012)
		Holliday and McFarland. (1996)

The role of carnosine improves exercise performance in human; the exercise produces free radicals of reactive oxygen species (ROS) in muscle cell led to muscle fatigue. Carnosine can inhibit the oxidation of ROS in skeletal muscle as show in Figure 2.5. Firstly, an ability to resist pH changes (proton buffering capacity) due to glycolysis processes in the anaerobic state of muscle cells produces H^+ and lactate ions, resulting in a pH of 6.5 or less, which is associated with muscle contraction, causing muscle fatigue. Secondly, the contraction strength of the muscle depends on the amount of calcium released by the sarcoplasmic reticulum. The role of carnosine is to regulate calcium release and stimulate the sarcoplasmic reticulum to release

calcium in the process of proper muscle contraction (Blancquaert et al., 2015). Thirdly, carnosine inhibits the oxidation of ROS. Fourthly, carnosine acts as a chelate that surrounds Cu^{2+}, preventing it from reacting. And fifthly, when carnosine exits, the muscle is broken down into histidine or histamine. Then those are absorbed into the bloodstream (Boldyrev et al., 2013). Therefore, the increase of carnosine improves exercise performance in humans.

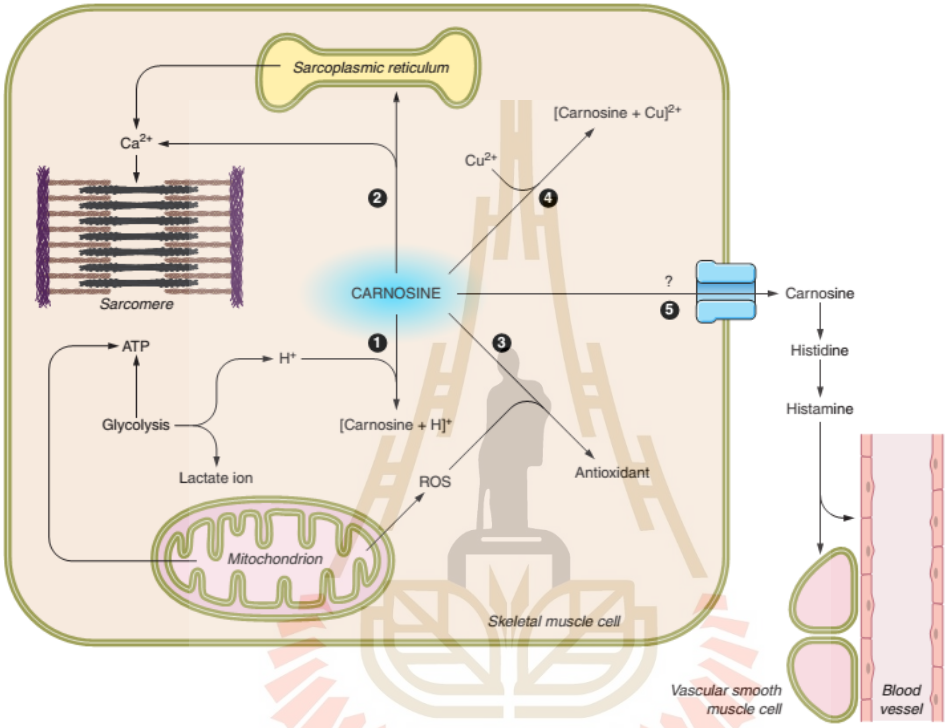

Figure 2.5 Role of carnosine in skeletal muscle: 1) proton buffering capacity; 2) regulatorof calcium release and calcium sensitivity; 3) protection against reactive oxygen species (ROS); 4) chelation of transition metal ions; and 5) extracellular provider of histidine/histamine.

Source: Boldyrev et al. (2013).

2.6 The role of carnosine on animals' industry

2.6.1 The role of carnosine on growth performance

From the research of Hu et al. (2009), the study of supplemented carnosine at 0 and 0.5% in broiler diets and Ma et al. (2010) supplemented carnosine at levels 0 to 100 mg/kg in pig diets, it was found that carnosine supplementation had no effect on growth performance. Kopec et al. (2016) reported that the increase

of carnosine by histidine supplementation at 0 and 0.18% in turkey diets had no effect on growth performance (P>0.01). Cong et al. (2017) a, supplemented carnosine at 0-400 mg/kg^{-1} in broiler diets and found that different levels of carnosine supplementation had no effect on the feed intake, growth rate, and feed conversion ratio (P>0.01), except for feed conversion ratio at 1-21 days. Qi et al. (2018), β-alanine supplementation at 0-2,000 mg/kg showed that the growth rate and feed conversion ratio increased significantly from the control group (P<0.01), but no significant difference on feed intake (P>0.01) (as shown in Table 2.3).

From the literature review, it is indicated that the improving of carnosine content by supplementation with carnosine synthesis substrate has no negative effect on growth performance. At the same time supplementing the suitable dosage and/or period may increase growth performance.

Table 2.3 The effects of L-histidine, β-alanine and carnosine supplementation on growth performance.

Specie	Supplementation	Day	Growth performance			Reference
			Feed intake (g)	ADG (g)	FCR	
Broiler	Carnosine (%)	0	1-42	92.4±3.3	45.9±1.5	2.01±0.9	Hu et al. (2009)
		0.50		94.0±5.8	47.9±3.3	1.96±0.0	
Pigs	Carnosine (mg/kg)	0	1-56	2560	814	3.15	Ma et al. (2010)
		25		2516	806	3.13	
		50		2534	811	3.12	
		100		2533	822	3.08	
Turkes	Histidine (%)	0	1-103	291	-	2.94	Kopec et al. (2016)
		0.18		295	-	2.79	
Broiler	Carnosine (mg/kg^{-1})	0	1-21	952	587	1.62^{a}	Cong et al. (2017)a
		100		906	584	1.55^{b}	
		200		914	588	1.55^{b}	
		400		922	598	1.54^{b}	

Table 2.3 (Cont).

Species	Supplementation		Day	Growth performance			Reference
				Feed intake (g)	ADG (g)	FCR	
	Carnosine	0	1-42	3773	2104	1.79	
	(mg/kg^{-1})	100		3717	2120	1.75	
		200		3706	2113	1.75	
		400		3712	2157	1.73	
Broiler	β-alanine	0	1-21	61.0	42.5**	1.44**	Qi et al.
	(mg/kg)	250		63.5	47.2**	1.34**	(2018)
		500		64.5	48.8**	1.32**	
		1,000		61.6	46.3**	1.33**	
		2,000		64.7	48.3**	1.34**	
	β-alanine	0	1-42	105	59.9*	1.75*	
	(mg/kg)	250		102	61.4*	1.67*	
		500		107	64.9*	1.63*	
		1,000		101	60.3*	1.67*	
		2,000		104	61.5*	1.69*	

ADG, average daily gain; FCR, feed conversion ratio.

$^{a-b}$ Mean within a column with no common superscript differ significantly ($P<0.05$)

* Significant different between group ($P<0.05$)

** Significant different between group ($P<0.01$)

2.6.2 The role of carnosine on meat quality

According to research by Hu et al. (2009), it was reported that carnosine supplementation at 0 and 0.5% in broiler diets increased meat yield when compared with the control group ($P<0.05$). Cong et al. (2017) a, and Cong et al. (2017) b, found that supplemented carnosine at 0-400 mg/kg-1 in broiler diets could increase a pH at 45 min, redness, and decrease drip loss, cooking loss, and shear force when compared with the control group ($P>0.05$).While Qi et al. (2018)studied β-alanine supplementation at 0-2,000 mg/kg and found that redness was increased at 24-min and 24-hour, but shear force was decreased ($P>0.05$).

In the majority of animals such as pigs or buffaloes, according to the report of Das et al. (2006), carnosine supplementation at 0-1.5% in the diet had an effect on pH at 45 minutes and increased water-holding capacity but decreased cooking loss. Ma et al. (2010) studied carnosine supplementation at a level of 0 to 100 mg/kg in pig diets and found that carnosine supplementation at different levels increased the pH at 45 min and redness but decreased the drip loss (as shown in Table 2.4).

This literature review demonstrates, that the increase of carnosine can improve the meat quality in animal industry, especially slowdown pH at 45 minutes, causing increased water-holding capacity.

Table 2.4 The effect of β-alanine and carnosine supplementation on meat quality.

Species	Supplementation	Organ	Result	Reference
Broiler	Carnosine	Breast	- meat yield ↑*	Hu et al. (2009)
Broiler	Carnosine	Breast	- pH 45 min ↑* - redness ↑* - drip loss ↓*	Cong et al. (2017)a
Broiler	Carnosine	Thigh	- pH 45 min, 24 h ↑* - redness ↑* - drip loss ↓* - cooking loss ↓* - shear force ↓*	Cong et al. (2017)b
Broiler	β-alanine	Breast	- shear force ↓* - redness 45 min, 24 h ↑*	Qi et al. (2018)
Buffalo	Carnosine	Mixed meat	- pH ↑* - water-holding capacity ↑* - cooking loss ↓*	Das et al. (2006)
Pig	Carnosine	Longissimus dorsi	- pH 45 min, 24 h and 48 h ↑* - drip loss at 48 h ↓* - redness value at 45 min ↑*	Ma et al. (2010)

* Significant different ($P<0.05$)

2.7 Factors affecting on carnosine content

From previous research papers, it was found that internal factors such as breeds and muscle type, and external factors such as age, sex, diet, and exercise and stress, influenced the amount of carnosine (as shown in Table 2.5).

Table 2.5 Factors affecting carnosine content.

Factor	Effect of carnosine content	Reference
Internal factors		
Genetics	Different between breed	Tian et al. (2007), Jung et al. (2013), Intarapichet and Maikhunthod. (2005)
Type of muscle fiber	White muscles > red muscles.	Tian et al. (2007), Kim et al. (2012), Cornet and Bousset (1999)
External factors		
Age	Age high amount of carnosine is low	Kim et al. (2012)
Sex	Female > male	Jung et al. (2013)
Feed	L-histidine supplementation, β-alanine supplementation	Tomonaga et al (2012), Kai et al. (2014), Kralik et al. (2015)
Exercise	Trained muscle > Untrained muscle	Bex et al. (2013)

2.7.1 Genetics factor

Chicken breeds influenced the amount of carnosine. According to Jung et al. (2013), it was found that red-brown native chickens have the highest amounts of carnosine when compared to chickens of other feather colors in both areas. At the same time, Intarapichet and Maikhunthod. (2005) reported that four hybrid breeds had the highest carnosine content in breast meat, while the hybrid breeds had the highest carnosine content in thigh meat.

In Black-boned Silky Flow, carnosine content was twice when compared with commercial broilers (Tian et al., 2007) as shown in Table 2.6. From different types of muscle fibers, it was found that the carnosine content of type IIB was higher than type IIA (Swell et al., 1992, Dunnett and Harris, 1995), while Black-boned Silky Flow has only type IIB (Nakamura et al., 2003) and broilers have two types of muscle fibers – IIR and IIW (Kojima et al., 2014).

Table 2.6 The amount of carnosine in black-boned silky flow and broilers.

Breed	Muscle	Carnosine	Anserine	Unit	Reference
Silky Fowl	Breast	798.3±85.9^{a}	631.8±12.4^{b}	(mg/100g)	(Kojima et al.,
	Thigh	288.2±20.4^{x}	261.9±9.4^{y}		2014)
Japanese Game	Breast	455.5±36.8^{b}	809.5±15.6^{a}		
Cross	Thigh	161.0±8.6^{y}	291.8±7.7^{xyz}		
	Breast	441.3±49.9^{b}	762.6±33.1^{a}		
HinaiJidori	Thigh	164.5±16.4^{y}	326.0±9.6^{x}		
	Breast	478.9±23.8^{b}	780.7±23.7^{a}		
Nagoy Breed	Thigh	126.7±13.3^{y}	277.2±8.8y^{z}		
	Breast	417.2±14.8^{b}	647.9±12.6^{b}		
Broiler	Thigh	183.4±14.7^{y}	254.9±7.6^{yz}		
Black-Bone Silky Fowl	Breast	1.6±0.3^{a}	-	(mg/g)	(Tian et al., 2007)
	Thigh	0.4±0.1^{x}	-		
White Plymouth Rock	Breast	0.9±0.4^{b}	-		
	Thigh	0.2±0.1^{y}	-		

$^{a-b}$ = significant between breed of breast meat

$^{x-y}$ = significant between breed of thigh meat

2.7.2 Type of muscle fibers

Muscle fibers are divided into four main types: type I (slow-oxidative), IIA (fast-oxidative), IIB, and IIX (fast-glycolytic). The IIB is found to have a high amount of carnosine compared to type IIA and type I, respectively (Cornet and Bousset, 1999).

This is consistent with the previous report that breast muscle comprises most of the IIB-type muscle fibers, which is a reason why there are higher amounts of carnosine than in another area (Intarapichet and Maikhunthod., 2005, Kim et al., 2012).

2.7.3 Age and sex factors

The amount of carnosine decreases when chickens get older (Kim et al., 2012), and Khumpeerawat et al. (2021) reported that age is a negative correlation with the expression of the ATPGD1 gene related to carnosine synthesis in slow-growing chickens (4-16 weeks of age). Females have a higher amount of carnosine than males (Jung et al., 2013 and Intarapichet and Maikhunthod., 2005).

2.7.4 Dietary factor

The carnosine content in the chicken meat can be increased by supplementing with dietary β-alanine and L-histidine from which the results show that L-histidine supplementation can increase higher carnosine content than supplementation with β-alanine. Moreover, the supplementation levels also affect carnosine accumulation in meat. Based on a study by Tomonaga et al. (2012), it is reported that supplementation with β-alanine can increase carnosine content in *Pectoralis superficialis* muscle and brains of 2-day chicks, of which the increasing is in accord with the level of β-alanine supplementation. Kralik et. al. (2015) studied β-alanine and L-histidine supplementation in the chicken diet and found that supplementation with β-alanine at lower than 0.5% resulted in no difference of carnosine content from the control group. However, carnosine content is the highest when supplementing with 0.5% L-histidine. As in research by Kai et al. (2014), L-histidine supplementation at 200% showed an increased carnosine concentration.

2.7.5 Exercise and stress factors

Carnosine can resist pH changes due to H^+ (proton buffering capacity) that occurs from the anaerobic glycolysis process in skeletal muscle cells arising from intense exercise, which is involved in a contraction of the muscles, causing the muscles to become fatigued. Meanwhile, Bex et al. (2013) found that carnosine increased with exercise compared to non-exercise individuals. Moreover, carnosine content in the breasts of stressed broilers under short-term heat stress is 10 times higher than that of nonstressed broilers (Manhiani et al., 20110). At the same time, increasing the stress of the animal resulted in the increased content of carnosine.

From the above mentioned, it is implied that carnosine content is varied by many factors especially genetics factor and diet; hence, the increase of carnosine synthesis in chicken by genetics improvement and environmental management is possible.

2.8 Digestion, transport, and synthesis of carnosine in muscles

Most studies of mechanisms involved the digestion, transport, and carnosine synthesis in muscles with focus on the role of carnosine on human health. Mechanism of carnosine synthesis in animal was firstly investigated in mouse by Everaert et al. (2013). As the following mechanisms, after carnosine is ingested, carnosine is absorbed into the intestinal by oligopeptide transporter 1 and 2 proteins (PEPT1 and PEPT2) located at the brush border membrane, and protons are also transported in this process. Carnosine is then broken down by Carnosinase into β-alanine and L-histidine and transported by amino acid transporters into blood vessels. However, little carnosine is absorbed into the bloodstream via peptide transporter as carnosine (Boldyrev et al., 2013). Then, both β-alanine and L-histidine are transported by amino transporters into muscle sarcoplasm and synthesized by carnosine synthase to be carnosine (Harris et al., 2012).

β-alanine is synthesized in the body through a lot of pathways in the liver and transported into the bloodstream. Blood vessels carry carnosine, β-alanine, and histidine into the muscle via a specific protein transporter. Then, the carnosine synthase enzyme in muscle synthesizes β-alanine and histidine into carnosine. Homocarnosine is synthesized by the binding L-histidine and Gamma-aminobutyric acid by using the same enzyme as carnosine, while anserine and opidine are caused by a specific enzyme carnosine-N-methylase. However, there is no previous reports on the enzymes involved in carcinine biosynthesis (Saunders et al., 2016, Miyaji et al., 2012, Everaert et al., 2013)). The remaining β-alanine, and histidine will be destroyed in the liver (Andersen et al., 2009), as shown in Figure 2.6.

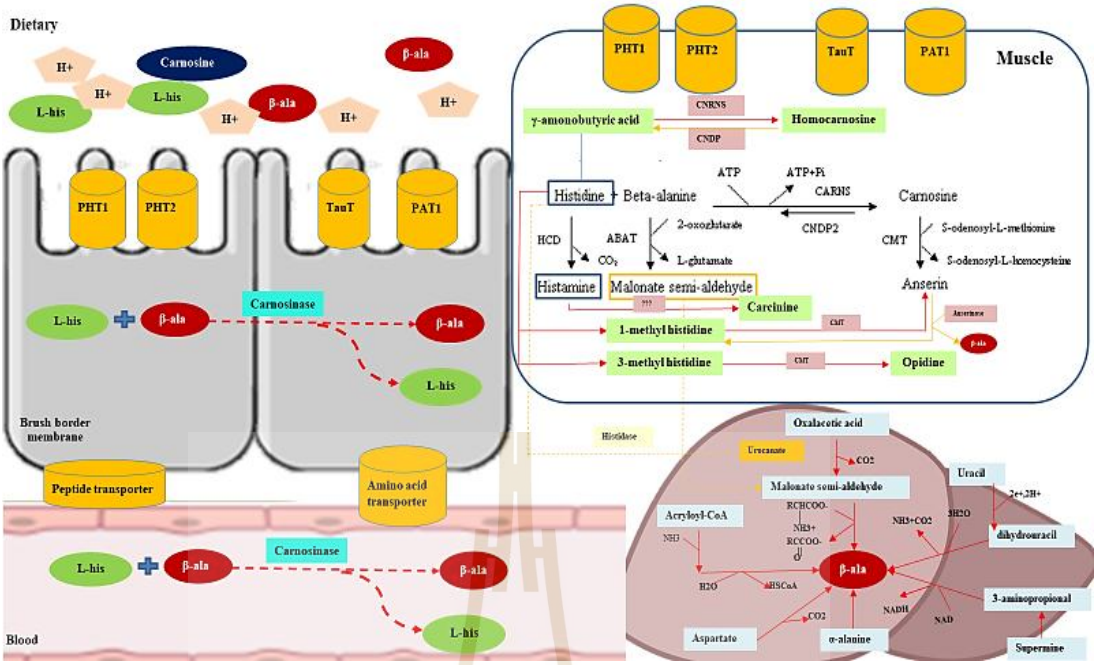

Figure 2.6 Pathway involved with carnosine and derivative synthesis in humans and mice. Adapted from: Boldyrev et al. (2013), Harris et al. (2012), Saunders et al. (2016), Miyaji et al. (2012), Everaert et al. (2013), Wang et al. (2014) and Andersen et al. (2009).

2.9 Gene and protein involved with carnosine synthesis in chicken

The most studies of gene involved with carnosine synthesis in chicken is the study about the supplementation with carnosine synthesis substrates on the expression of gene involved with carnosine-related enzyme and transporters. According to reports from Cong et al. (2017) b, β-alanine supplementation in the chicken diet at 0-2,000 mg/kg diet for 42 days increased the amount of carnosine in the muscle. And also genes involved in β-alanine transport and carnosine-related genes were significantly higher than the control group (P>0.05). Consistent with Qi et al. (2018), supplementing carnosine in the chicken diet at 0-400 levels mg/kg diet for 42 days increased the amount of carnosine in the muscle and the gene expression involved in carnosine synthesis was higher increased than the control group (P>0.05). Moreover, Qi et al. (2021) also reported that the supplementation with L-histidine can activate the expression of gene involved with carnosine synthesis such as histidine decarboxylase (HDC), proton-coupled oligopeptide transporters (PEPT1) and carnosine

synthase (CARNS) in fast-growing chicken. From the compilation of the above research papers, it was found that β-alanine, carnosine and L-histidine supplementation had activated the expression of the genes involved in carnosine transport, absorption, and synthesis as well as increase of carnosine synthesis in chicken.

Regarding the studies of protein, over the years, there have been few reports of studies on proteins involved in carnosine synthesis and derivatives in chicken. (as shown in Table 2.7). From the study by Drozak et al. (2010) on extracted carnosine synthase from chicken breast meat, it was found that this enzyme was identified as ATP-grasp Domain-containing Protein 1 (ATPGD1). One of the natural carnosine derivatives, anserine, is formed by the presence of methyl groups in the compound. Drozak et al. (2013) studied by extracting carnosine N-methyltransferase from chicken breast meat and found three important polypeptides related to carnosine N-methyltransferase, which could be identified to histamine N-methyltransferase-like (HNMT-like) protein that demonstrated to stimulate anserine production.

However, the expression studies to detect and quantify messenger RNA (mRNA) levels of specific genes, as well as a specific protein, cannot make an understanding about a set of actions or interactions between genes and their products that results in the formation or change of some components of the system, essential for the correct functioning of a biological system (Segundo-Val and Sanz-Lozano, 2016). Unfortunately, holistic molecular mechanisms related to carnosine synthesis and other consequences in slow-growing chicken are not yet revealed.

Table 2.7 The study of gene and protein expansion involved with carnosine synthesis in meat.

Specie	Condition	Organ	Result	Reference
Broiler	Carnosine	Thigh	- carnosine content ↑* - CARNS1 gene expression ↑*	Cong et al. (2017)b
Broiler	β-alanine Supplementation	Breast	- carnosine content ↑* - CARNS1 gene expression ↑* - SLC6A6 gene expression ↑*	Qi et al. (2018)
Broiler	L-histidine Supplementation	Breast	- carnosine content ↑* - HDC↑* - PEPT1 ↑* - CARNS ↑*	Qi et al. (2021)
Broiler	Extract Carnosine synthase	Breast	- Identified ATP-grasp domain-containing protein	Drozak et al. (2010)
Broiler	Extract Carnosine N-methyltransferase	Breast	- Identified Histamine N-methyltransferase-Like protein	Drozak et al. (2013)

* Significant difference ($P<0.05$)

CARN1, carnosine synthase; SLC6A6, β-alanine transporter; ATPGD1, ATP-grasp domain-containing protein-1; TauT, beta-alanine transporters; ABAT, beta-alanine transaminase; HDC, histidine decarboxylase; PEPT1, proton-coupled oligopeptide transporters; CARNS, carnosine synthase

2.10 Fourier transform infrared (FTIR)

FTIR is the method of infrared spectroscopy that is used to obtain infrared spectrum of transmission or absorption of samples (solid, liquid, and gas). The resulting spectrum of both organic and inorganic chemicals in each sample is

determined depending on the infrared absorption frequency range 600–4000 cm^{-1}. The specific molecular groups prevailing in the sample will be determined through spectrum data in the automated software of spectroscopy (Shameer and Nishath., 2019).

Recent findings give further suggest that FTIR spectrometry has been used to study the relative effectiveness of biochemical change on the physicochemical of meat characteristics change (Bocker et al., 2007; Candogan et al., 2020). For example, Beattie et al. (2004) suggested that shear force, tenderness, and texture of beef are changed when biochemicals compound such as the ratio of α-helices to β-sheet and the hydrophobicity of the myofibrillar in environment changes. Katemala et al. (2021) reported that the relative content of β-sheet is positively correlate with the shear force of KRC meat. Moreover, FTIR spectrometry has been used to study meat quality in terms of biochemical changes (Bocker et al., 2007; Candogan et al., 2020).

Synchrotron radiation-based Fourier transform infrared (SR-FTIR) microspectroscopy is a highly powerful and sensitive approach for detecting the vibrations of molecules that provide information about protein secondary structure, lipids, and glycogen. This technique is advantages to analyze samples at the micron level or micro-sample areas. (Wang et al., 2015). The advantage of synchrotron light brightness (which is usually 100–1000 times brighter than a conventional globar source and has a small effective source size), SR-FTIR microscopy can preserve the crystal structure and morphology of the samples while exploring the molecular chemistry within the microstructures of samples with a high signal–to–noise ratio (S/N) at ultraspatial resolutions (Pascolo et al., 2014). Yu, 2004 suggested that the SR-FTIR technique can be used for feed science and animal nutrition research. Therefore, compared to the results of FTIR spectrometry, the use of synchrotron-FTIR to measure the transformation of biochemical compounds on the physicochemical of meat characteristics change was expected to produce more interesting findings.

2.11 Proteomic technique

Proteomics is the study of protein molecules throughout the system and also includes the expression of proteins that are transcribed from the DNA strands at the cellular level, tissues and components. Because proteome differs from cell to cell

and from time to time, which a result of proteins are essential to perform various functions in living organisms, the proteomics techniques are highly effective in the studies involving the effect of food on organism function (Guerin-Dubiard et al., 2006).

The study in mRNA level limited to demonstrate a directly correlated to actual protein abundance, because it needs to be modified before it is translated into proteins. As a result, one gene can be coded into multiple proteins because one gene is one peptide, while the protein consists of combined multiple peptides (Sentandreu et al., 2010). However, an arise of phenotypic diversity from a set of common genes is mostly determined by proteins and their enzymatic biological functions, which posttranslational modifications promote the regulation of structure, function, localization, maturation, and turnover of proteins (Rifai et al., 2006).

There is currently research reporting on the proteome and its related-metabolic pathways in different conditions of chicken. Teltathum and Mekchay. (2010), reported that PKM2, PGAM1, and TPI1 protein were related to the tenderness characteristics of the meat. While Phongpa-Ngan et al. (2011) reported that the proteins creatine kinase, pyruvate kinase, triosephosphate isomerase and ubiquitin of Thai indigenous chickens were associated with water holding capacity in chicken meat. In fast-growing chicken, Lee and Choi. (2021) reported that heat shock proteins 90 (HSP90) is associated with the breast quality characteristics of PSE-like condition. In terms of meat color, Sayd et al. (2006) reported that the protein HSP27, αB-crystallin and Glucose-regulated protein were associated with pig meat color. These evidents demonstrate that the finding of protein groups associated with the trait of interest could lead to a gene marker for use in animal selection to meet the desired characteristics.

Therefore, the selection of proteomics approach in this research was appropriate complement to genomic data, better understanding of the mechanisms of carnosine synthesis and its derivatives at the molecular level.

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CHAPTER III

EFFECTS OF β-ALANINE AND L-HISTIDINE SUPPLEMENTATION ON CARNOSINE CONTENTS IN AND QUALITY AND SECONDARY STRUCTURE OF PROTEINS IN SLOW-GROWING KORAT CHICKEN MEAT

3.1 Abstract

Carnosine enrichment of slow-growing Korat chicken (**KRC**) meat helps differentiate KRC from mainstream chicken. We aimed to investigate the effects of β-alanine and L-histidine supplementation on the carnosine synthesis in and quality and secondary structure of proteins in slow-growing KRC meat. Four hundred 21-day-old female KRC were used, and a completely randomized design was applied. The chickens were divided into 4 experimental groups: basal diet (A), basal diet supplemented with 1.0% β-alanine (B), 0.5% L-histidine (C), and 1.0% β-alanine combined with 0.5% L-histidine (D). Each group consisted of 5 replicates (20 chickens per replicate). On day 70, 2 chickens per replicate were slaughtered, and the levels of carnosine, anserine, and thiobarbituric acid reactive substances were analyzed. Biochemical changes were monitored using synchrotron radiation-based Fourier transform infrared microspectroscopy; 5 chickens per replicate were slaughtered, and the meat quality was analyzed. Statistical analysis was performed using ANOVA and principal component analysis (**PCA**). Group D chickens exhibited the highest carnosine meat content, followed by those in groups B and C. However, amino acid supplementation did not affect anserine content and growth performance. Higher carnosine levels correlated with increasing $pH_{45\ min}$ and decreasing drip loss, cooking loss, shear force, and lipid oxidation. PCA revealed that supplementation with only β-alanine or L-histidine was related to increased content of β-sheets, β-turns, and aliphatic bending groups and decreased content of α-helix groups. This study is the first to report such findings in slow-growing chicken. Our findings suggest that KRC can synthesize the highest carnosine levels after both β-alanine and L-histidine supplementation. Higher carnosine contents do not adversely affect meat quality,

improve meat texture, and alter the secondary structures of proteins. The molecular mechanism underlying carnosine synthesis in chickens needs further study to better understand and reveal markers that facilitate the development of nutrient selection programs.

Key words: carnosine, β-Alanine, L-Histidine, slow-growing chicken, synchrotron radiation-based Fourier transform infrared microspectroscopy

3.2 Introduction

Korat chicken (**KRC**) is a crossbreed between Thai indigenous Leung Hang Khao sires and Suranaree University of Technology synthetic breed dams. The main purpose of the crossbreeding was to provide an alternative breed to promote the occupation of smallholder farmers in Thailand and Southeast Asia. Korat chicken is categorized as a slow-growing chicken (Poompramun et al., 2021) because it has a lower growth rate than typical chicken. Its body weight reaches market weight (approximately 1.2 kg to 1.3 kg) within 9 to 10 wk of age (Hang et al., 2018). The lower performance of KRC, particularly of female KRC, is a significant disadvantage for farmers.

Because KRC is a crossbred chicken, half of its genetic background comes from the Thai indigenous chicken. Hata et al. (2021) concluded that Thai and most other indigenous chickens in South-East Asia and southern China originate from the same ancestor and were also selected from a similar environment. Hence, KRC could be used as a model for slow-growing chickens.

Sex is an important factor influencing carnosine content in chicken meat (Intarapichet and Maikhunthod, 2005). Previous studies reported that female chicken meat accumulates higher carnosine content than male chicken meat (Jung et al., 2013; Intarapichet and Maikhunthod, 2005). Carnosine is a major histidine-containing dipeptide consisting of β-alanine and L-histidine (Barbaresi et al., 2019). Anserine, a derivative of carnosine, is composed of β-alanine and 3–methyl histidine (Drozak et al., 2013). Carnosine and anserine play a very important role in human health by protecting and relieving pain from diseases such as aging, cancer, Alzheimer's disease, Parkinson's disease, and the complications of type-2 diabetes (Hipkiss et al., 2013).

Therefore, enriching carnosine in KRC is a good strategy to improve the competitiveness of smallholder farmers and differentiate KRC from commercial broilers.

Carnosine synthesis and its content in muscle can be enhanced when amino acid substrates are supplemented in the diet (Kopec et al., 2020; Kralik et al., 2015; Qi et al., 2018). Regarding growth performance, the role of carnosine and its substrate (β-alanine and L-histidine), including other related factors, has been investigated in fast-growing chickens. Van Vught et al. (2008) found that histidine promotes growth hormone secretion, whereas Bhattacharya et al. (2015) reported that β-alanine could enhance physical fitness, and Tiedje et al. (2010) reported that β-alanine could act as a neurotransmitter to regulate the secretion of hormones related to growth and development. In addition, there are many studies related to the effect of carnosine and its substrates on growth performance in fast-growing chicken, some of which found significant effects (Cong et al., 2017b; Kopec et al., 2020; Qi et al., 2018), but that of Hu et al. (2009) did not. Regarding meat quality, Kralik et al. (2015) reported that supplementation of a higher amount of β-alanine or L-histidine in the diet could increase carnosine content in chicken breast meat, but does not affect thiobarbituric acid reactive substances (TBARS). Cong et al. (2017a) found that chickens that were fed a carnosine-supplemented diet could produce high-quality meat by increasing $pH_{45\ min}$, drip loss, and cooking loss. Dietary β-alanine supplementation reduces the shear force of the meat in broiler chicks (Qi et al., 2018). Kralik et al. (2018) reported that β-alanine and L-histidine supplementation combined with magnesium oxide decreased $pH_{45\ min}$ and drip loss. Unfortunately, data on varying metabolism (Sirri et al., 2011), movement behavior (Castellini et al., 2016), and ability to resist oxidative stress (Mattioli et al., 2017) in slow-growing chickens are limited compared to those on fast-growing chickens. To date, the effect of substrates of carnosine synthesis and its content in the muscle on growth performance and meat quality in slow-growing chickens remains unclear.

Regarding meat quality changes, Beattie et al. (2004) suggested that shear force, tenderness, and beef texture are influenced by changes in biochemical compounds such as the ratio of α-helices to β-sheet and the hydrophobicity of the myofibrils in the environment. Katemala et al. (2021) demonstrated that the relative content of β-sheet is positively correlated with the shear force of KRC meat. Moreover, Fourier

transform infrared (**FTIR**) spectrometry has been used to study meat quality in terms of biochemical changes (Bocker et al., 2007; Candoğan et al., 2020). Synchrotron radiation-based Fourier transform infrared (**SR-FTIR**) microspectroscopy is a powerful and sensitive approach for detecting the vibrations of molecules that provide information about protein secondary structure, lipids, and glycogen. This technique can be used to analyze samples at the micron level or micro-sample areas (Wang et al., 2015). Yu (2004) suggested that the SR-FTIR technique can be used for feed science and animal nutrition research. Therefore, the results using synchrotron-FTIR to measure the transformation of biochemical compounds in our study is expected to be more interesting than those using FTIR spectrometry.

The goal of this research was to increase carnosine synthesis in KRC using genetic manipulation. We aimed to elucidate the adverse effects of carnosine content on growth performance and meat quality. Hence, in this study, an experiment was designed using β-alanine and L-histidine supplementation (substrate of carnosine synthesis) to increase carnosine levels in KRC breast meat. Kralik et al. (2015) identified that supplementation with 1% β-alanine or 0.5% L-histidine significantly increased carnosine content in chicken breast meat (20.48 % and 25.96%, respectively) but did not affect its lipid oxidation. Therefore, we investigated carnosine synthesis in slow-growing KRC that were fed 1% β-alanine and 0.5% L-histidine-supplemented diet and monitor the effect of supplementation on growth performance and meat quality, including biochemical compounds and secondary structure of proteins in meat using SR-FTIR microspectroscopy. To the best of our knowledge, this is the first study on the effect of dietary supplementation on biochemical and physiochemical changes in slow-growing chicken meat. Our findings will provide insights into carnosine synthesis and its effect on chicken meat properties before designing a breeding program in slow-growing chickens.

3.3 Materials and methods

3.3.1 Experimental Design and Chicken Handling

Female KRC produced in the Suranaree University of Technology farm were used for the study. When the chickens were 21 days old, they were randomly assigned to 4 experimental diet groups using a completely randomized design with 5

replicates per group and 20 chickens per replicate. The mean and SD of the chicken body weight was approximately 266.04±3.03 g.

The 4 experimental diets, all of which were formulated based on the National Research Council nutrient recommendations (1994), were as follows: basal diet (A), basal diet supplemented with 1.0% β-alanine (B), 0.5% L-histidine (C), and 1.0% β-alanine combined with 0.5%L-histidine (D). The ingredient and nutrient compositions of the experimental diet for growers (22 to 42 days) and finishers (43 to 70 days) are shown in Table 3.1. Analytical value of amino acids content (%) in experimental diets in different growing phases shown in Table S1.

The chickens were raised in an open house, and the stocking density was 8 birds/m^2. Food and water were provided *ad libitum*. The vaccination program was performed as per guidelines set by the Department of Livestock Development, Bangkok, Thailand.

The chickens were weighed, feed intake data were collected weekly, and data were used to calculate the body weight gain, average daily feed intake, average daily gain, and feed conversion ratio at 21, 42, and 70 days.

3.3.2 Sample Collection

On day 70, 10 chickens per group (2 chickens/ replicate) were randomly tagged and stunned by chloroform. Then chickens were slaughtered by decapitation and bled. Approximately 10 g of breast meat (*M. pectoralis major*) was packed in a vacuum bag and frozen at -80°C. Samples were analyzed for carnosine, anserine, and TBARS. The other portion (10 g) was kept in a zip-lock bag, stored at 4°C for 24 h, and used for biochemical analysis.

Five chickens per replicate were randomly tagged and fasted for 24 h. They were then stunned by electric shock, decapitated, bled, scalded before mechanical de-feathering, and manually eviscerated. Carcasses were chilled at 4°C for 24 h, and then the breast meat was removed for meat quality measurement.

Table 3.1 Ingredient and nutrient composition of experimental diet in different growing phase.

	Grower (22 to 42 days)[1]				Finisher (43 to 70 days)[1]			
	A	B	C	D	A	B	C	D
Ingredients (kg)								
Corn	59.40	57.52	58.30	56.64	67.30	65.14	66.14	64.44
Soybean meal (44% CP)	30.80	28.40	28.22	28.78	26.30	26.70	26.50	26.70
Full fat soybean	2.42	5.50	5.80	5.20	0.00	0.00	0.00	0.00
Rice bran oil	4.00	4.20	3.80	4.50	3.04	3.80	3.50	4.00
L-Lysine	0.18	0.18	0.18	0.18	0.19	0.19	0.19	0.19
DL-Methionine	0.21	0.21	0.21	0.21	0.14	0.14	0.14	0.14
Salt	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Calcium carbonate	1.42	1.42	1.42	1.42	1.20	1.20	1.20	1.20
MDCP (P21)	1.02	1.02	1.02	1.02	1.28	1.28	1.28	1.28
Premix[2]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
β-Alanine	0	1.00	0	1.00	0	1.00	0	1.00
L-Histidine	0	0	0.50	0.50	0	0	0.50	0.50
Total (kg)	100	100	100	100	100	100	100	100
Calculated composition (%)								
ME (kcal/kg)	3,113	3,116	3,112	3,111	3,112	3,116	3,118	3,110
Crude protein, %	19.39	19.30	19.39	19.29	17.08	17.10	17.08	17.04
Crude fiber, %	3.60	3.56	3.58	3.55	3.32	3.30	3.31	3.29
Ether extract, %	6.94	7.60	7.29	7.82	5.81	6.49	6.23	6.66
Calcium, %	0.90	0.90	0.90	0.90	0.86	0.86	0.86	0.86
Total phosphorus, %	0.57	0.57	0.57	0.56	0.60	0.59	0.59	0.59
Histidine	0.45	0.48	0.99	0.98	0.37	0.37	0.87	0.87

[1]Treatment groups are A (control), B (supplemented with 1.0% β-alanine), C (supplemented with 0.5% L-histidine), and D (supplemented with 1.0% β-alanine + 0.5% L-histidine) respectively.

[2]Premix (0.5%) provided the following per kilogram of diet: 15,000 IU of vitamin A, 3000 IU of vitamin D3, 25 IU of vitamin E, 5 mg of vitamin K3, 2 mg of vitamin B1, 7 mg of vitamin B2, 4 mg of vitamin B6, 25 ug of vitamin B12, 11.04 mg of pantothenic

acid, 35 mg of nicotinic acid; 1 mg of folic acid, 15 μg of biotin, 250 mg of choline chloride, 1.6 mg of Cu, 60 mg of Mn, 45 mg of Zn, 80 mg of Fe, 0.4 mg of I and 0.15 mg of Se.

3.3.3 Meat Quality Measurement

pH. The breast meat pH (25 samples/treatment) was measured using an electronic pH meter (UltraBasic pH meter, Model UB10, Denver Instrument, Bohemia, NY, USA) at 45 min and 24 h postmortem. The pH was measured 3 times in the same area, and the probe was washed with ultrapure water between different sample measurements.

Drip Loss. At 24 h postmortem, drip loss was calculated using the method establish by Kralik et al. (2018). The breast meat was cut in 2 × 3 cm pieces with an approximate weight of 7 g, mopped, and weighed before being kept in a plastic bag at 4°C for 24 h. Then, the sample was mopped and reweighed to calculate drip loss using the following equation:

$$\text{Drip Loss (\%)} = [(\text{initial weight} - \text{final weight})/\text{initial weight}] \times 100\%$$

Cooking Loss. Cooking loss was determined using the method described by Kim et al. (2016) with slight modifications. The breast meat was cut in pieces of approximately 2×3×1 cm^3 from the same location and weighed. Samples were cooked in a water bath at 80°C until the core temperature reached 71°C. The samples were then cooled at room temperature for 3 h and weighed. The percentage of cooking loss was calculated using the following equation:

$$\text{Cooking loss (\%)} = [(\text{initial weight} - \text{cooked weight})/\text{initial weight}] \times 100\%$$

Shear Force. The shear force of cooked samples (2×1×1 cm^3) was determined following the method of Kim et al. (2016) using a Texture Analyzer (TA-XT Plus, Stable Micro System Ltd., Surrey, UK). A Warner-Bratzler shear attachment was used at a test speed of 2 mm/s. The samples were cross-sectionally cut into muscle fibers. The shear force values of 25 samples per treatment were recorded.

3.3.4 TBARS

Breast meat stored at -80°C for 5 mo was used for TBARS measurements according to the method described by Kong et al. (2008) with slight modifications. A ground sample (1 g) was homogenized with 3 mL 7.5% trichloroacetic acid (w/v). The homogenate was centrifuged at 10,000 × *g* and 4°C for 10 min. One milliliter of the supernatant was added to 1 mL of 0.02 M thiobarbituric acid, and incubated at 95°C for 20 min. Subsequently, the sample was cooled at 5°C for 5 min, and the absorbance was measured at 532 nm using a BioTek Epoch Microplate Spectrophotometer (Epoch, BioTek, VT, USA). Malonaldehyde bis (diethyl acetal) (ACROS Organics™, Gothenburg, Sweden) was used as a standard.

3.3.5 Carnosine and Anserine Measurements

The carnosine and anserine contents were determined using the method of Mora et al. (2007) with slight modifications. Breast meat (0.1 g) was homogenized with 900 µL of 0.01 N HCl for 2 min and centrifuged at 12,000 × *g* at 4°C for 10 min. Supernatants were filtered through a 0.45-µm syringe filter. The filtrate (250 µL) was mixed with 750 µL of acetonitrile and stored at 4°C for 20 min. Then, the sample was centrifuged at 10,000 rpm at 4°C for 10 min, filtered through a 0.2-µm syringe filter, and stored at -20°C until use. Carnosine and anserine were separated on an Atlantis HILIC silica column (4.6×150 mm, 3 µm, Waters Corporation, Milford, MA) equipped with high-performance liquid chromatography (HPLC 1260, Agilent Technology, Santa Clara, CA). Mobile phase A containing 0.65 mM ammonium acetate in 75% acetonitrile at pH 5.5 and mobile phase B containing 4.55 mM ammonium acetate in 30% acetonitrile at pH 5.5 were used. The separation conditions were determined by the linear gradient of phase B from 0% to 100% for 13 min at 1 mL/min. Twenty microliters of the sample obtained after the addition of acetonitrile were injected. Dipeptides were detected at a wavelength of 210 nm. Quantification was performed using the external standard carnosine and anserine (Sigma-Aldrich, St. Louis, MO, USA) at 25°C.

3.3.6 SR-FTIR Microspectroscopy

After chilling, 10 breast meat samples from each group were cross-sectionally cut into a muscle fiber size of 1×1 cm and placed into an aluminum foil block. The frozen tissues were cut using a cryostat (Leica CM1950, Leica Biosystems

Nussloch GmbH, Nussloch, Germany) at a thickness of 7 μm and placed on the IR window (Crystran Ltd, Dorset, UK), then placed in a vacuum desiccator for 2 to 3 days before the SR-FTIR measurement.

Two muscle sections of each chicken were subjected to spectra analysis. FTIR spectra were measured at BL4.1 Infrared spectroscopy & Imaging, Synchrotron Light Research Institute, using the SR-FTIR spectrometer with a synchrotron light source in the mid-IR region. Spectra were collected on a Bruker FTIR spectrometer (Vertex70, Bruker Optics, Ettlingen, Germany) coupled to a Bruker Hyperion 2000-IR Microscope (Bruker Optik GmbH, Ettlingen, Germany) with a 36x objective, coupled to an MCT detector cooled with liquid nitrogen covering a measurement range from 4000 to 800 cm^{-1}. The FTIR spectra were obtained in the transmission mode, collecting 64 scans with a 10 × 10 μm aperture size at a resolution of 6 cm^{-1} over a measurement range from 4000 to 800 cm^{-1}. Each group comprising 400 spectra (20 spectra × 2 muscles replicates × 10 chickens) were processed using the OPUS 7.5 software (Bruker Optics Ltd., Ettlingen, Germany).

The integral areas were determined using second-derivative processing at the spectral regions from 3000 to 900 cm^{-1}, including lipid, amide I, amide II, CH-binding of lipid, amide III, and glycogen.

Curve fitting of amide I was determined using the original spectra after preprocessing to calculate the integral areas of amide I (1700 to 1600 cm^{-1}), α-helix (1644, 1655 cm^{-1}), β-sheet (1630 cm^{-1}), β-turn (1670 cm^{-1}), and antiparallel (1689 cm^{-1}) regions based on Gaussian and Lorentzian functions.

3.3.7 Statistical Analysis

Principal Component Analysis (PCA). Principal component analysis was used to identify the biochemicals in the spectral ranges from 3000 to 2800 cm^{-1} and 1800 to 900 cm^{-1}. All spectral data were preprocessed using the Savitzky-Golay algorithm for second derivative transformations at 13 smoothing points and normalized with extended multiplicative signal correction using the Unscrambler® X Multivariate Data Analysis software (version 10.1, Camo Analytics, Oslo, Norway). The 400 spectra were averaged into 20 spectra per treatment, and outliers were removed until 5 spectra per treatment for groups or clusters were obtained using PCA. The bi-plot correlation was used to represent the clustered differentiation of data, and

related variables were recalculated using the two-dimensional scatter plot of PCA with the predominant spectral range. The high loading SR-FTIR spectra were selected for multivariate analysis with dipeptide content and physicochemical properties. Data for all variables were weighted using an SD weighting process, and the relationship between variables was investigated using PCA bi-plot correlation.

Significant Difference Analysis. ANOVA was used to analyze the effect of experimental diets on growth performance, meat quality, TBARS, dipeptide content, the ratio of integral area, and secondary structure ratio. Significant differences between the means of the treatments were determined using Tukey's multiple tests. A p-value of <0.05 was considered statistically significant. SPSS Version 16.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

3.4 Results and discussion

3.4.1 Carnosine and Anserine Contents in KRC Breast Meat

The carnosine and anserine contents in KRC breast meat are presented in Table 3.2. The carnosine content was the lowest in the control group. Chickens fed a diet supplemented with β-alanine (B), L-histidine (C), or both amino acids (D) showed higher carnosine contents (P<0.05). The highest level of carnosine was found in group D, with a 52.8% increase compared to that of the control group. However, amino acid supplementation had no significant effect on the anserine content (P> 0.05).

Table 3.2 Carnosine and anserine contents in KRCs breast meat.

Parameter	Treatment group[1]				SEM[2]	P-value
	A	B	C	D		
Carnosine (μg/g)	2,756.6[c]	3,484.6[b]	3,659.8[b]	4,212.5[a]	82.88	<0.001
Anserine (μg/g)	10,577.2	10,391.6	10,312.7	10,272.8	282.47	0.88

Results were averaged from 10 chickens per treatment.

[a-c] Mean values with different superscripts in the same row indicate significantly different at P-value<0.05

[1]Treatment groups are A (control), B (supplemented with 1.0% β-alanine), C (supplemented with 0.5% L-histidine), and D (supplemented with 1.0% β-alanine + 0.5% L-histidine) respectively.
[2]SEM indicates standard error of mean

The results revealed that KRC could synthesize carnosine at approximately 2.76 mg/g. Kojima et al. (2014) reported that the carnosine content in the breast meat of 79-wk-old female Black Bond Silky Fowls was approximately 7.98 mg/g. Jung et al. (2013) reported that the carnosine content in the 20-wk-old breast meat from 5 lines of female Korean native chicken was approximately 1.69–1.83 mg/g, whereas Khumpeerawat et al. (2021) reported that the carnosine contents in the meat of 84-day-old black Chinese and KU Phupan chickens were approximately 5.01, and 5.27 mg/g, respectively. Fortunately, the carnosine level that KRC can synthesize is in the middle range of slow-growing chickens.

Carnosine synthesis can be improved when the chickens are fed carnosine synthase substrates. The carnosine content in the meat of chickens in groups B and C, supplemented with only β-alanine or L-histidine, respectively, was higher than that in the control group (26.42% and 32.76%, respectively). Regarding β-alanine supplementation, our results align with many previous studies (Kralik et al., 2015; Tomonaga et al.,2006). As demonstrated by Qi et al. (2018), β-alanine promotes the expression of carnosine-related transporters, and carnosine synthase increases the carnosine content. Carnosine synthesis based on the combined use of supplemented L-histidine and available β-alanine in the blood and muscle (Kai et al., 2015) can explain the observed increase in carnosine content when the diet was only supplemented with L-histidine. Moreover, the synthesis of carnosine may be one of the mechanisms by which animals try to control the L-histidine content balance in body.

The highest carnosine content was found when the diet was supplemented with both amino acids, consistent with the hypothesis that both amino acids are limiting amino acids for carnosine synthesis (Kai et al., 2015; Qi et al., 2018). This result, however, contrasted with that of Kopec et al. (2020), showing that the carnosine content did not differ compared to the results obtained using only β-alanine or L-histidine. Two possible reasons may explain these contrasting results.

First, Barbaresi et al. (2019) reported that in fast-growing broilers, the available L-histidine is primarily directed towards muscle protein synthesis, but in slow-growing chickens, the synthesis of anserine and carnosine may have been favored by higher availability of L-histidine. Second, slow-growing chickens may have a better ability to store carnosine in the muscle. Conversely, slow-growing chickens are resistant to oxidative stress, as confirmed by Lengkidworraphiphat et al. (2020).

When compared with the other groups, a significant difference in the anserine content was not found in the L-histidine supplementation group or the combined amino acid supplementation group. These results may be explained by the fact that anserine can be synthesized via 2 pathways: carnosine methylation (Boldyrev and Severin, 1990) or 3-methyl histidine and β-alanine synthesis (Drozak et al., 2013). Methionine is converted to S-adenosylmethionine that is a common co-substrate for supplying methyl group in metabolic process (Ramadan et al., 2021). Therefore, it is possible that when methionine is scarce, it is prioritized for protein synthesis before being used for other functions, including the transfer to methyl group and binding with L-histidine to generate anserine.

3.4.2 Carnosine Content on Growth Performance

KRC performance was expected to improve when the diet was supplemented with carnosine synthase substrates; however, contrary to our expectation, the results showed no significant difference in performance, including feed intake, body weight gain, average daily feed intake, average daily gain, body weight, and feed conversion ratio, among the different groups in each growth phase ($P>0.05$; Table 3.3).

It has been reported that oxidative stress affects poultry production (Surai and Fisinin, 2016). Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) levels and anti-oxidant activity related to environmental factors (sunlight, thermal irradiation, air temperature, humidity, and movement) and animals characteristics (species, gender, and rate of metabolism). Increase in ROS can decrease productive performance (Surai et al., 2019). This is in agreement with the study by Fouad et al. (2016), who reported that the increase of ROS leads to lipid peroxidation of intestine and pancreas cell walls, negatively affecting nutrient digestion and absorption. Lin et al. (2004) reported that the

oxidative stress-induced effect of corticosterone in chicken is related to a reduction in body weight and poorer feed efficiency. However, slow-growing chickens can tolerate stress (Mattioli et al., 2017), and these chickens may not require carnosine, which acts as an anti-oxidant. For this reason, we could not detect significant differences in performance.

Table 3.3 Effect of amino acids supplementation on growth performance of KRCs.

Parameter	Treatment group[1]				SEM[2]	P-value
	A	B	C	D		
22-42 d						
FI (g)	1018.64	1092.50	1037.32	1038.99	18.67	0.07
BWG (g)	381.97	386.21	382.48	390.17	5.68	0.72
ADFI (g)	48.51	52.02	49.40	49.47	0.89	0.07
ADG (g)	18.19	18.39	18.21	18.58	0.27	0.72
BW 42 d (g)	635.97	643.21	637.98	644.17	6.10	0.74
FCR	2.67	2.83	2.71	2.66	0.05	0.15
43-70 d						
FI (g)	1960.78	1940.13	1964.78	1933.38	59.71	0.98
BWG (g)	512.64	501.04	523.21	562.64	24.05	0.33
ADFI (g)	70.03	69.29	70.17	69.05	2.13	0.98
ADG (g)	18.31	17.90	18.69	20.09	0.89	0.33
BW 70 d (g)	1148.61	1144.25	1161.19	1206.81	26.01	0.34
FCR	3.85	3.91	3.77	3.46	0.19	0.38
22-70 d						
FI (g)	2979.42	3032.63	3002.10	2972.37	68.99	0.93
BWG (g)	894.61	887.25	910.69	952.81	25.65	0.30
ADFI (g)	60.80	61.89	61.27	60.66	1.41	0.93
ADG (g)	18.26	18.11	18.48	19.45	0.52	0.30
FCR	3.34	3.43	3.32	3.13	0.12	0.36

Results were averaged from 25 chickens per replicate, 100 chickens per treatment.

[1]Treatment groups are A (control), B (supplemented with 1.0% β-alanine), C (supplemented with 0.5% L-histidine), and D (supplemented with 1.0% β-alanine + 0.5% L-histidine) respectively.

[2]SEM indicates standard error of mean (mean ± SEM; n = 5/treatment).

FI, feed intake; BWG, body weight gain; ADFI, average daily feed intake; ADG, average daily gain; BW, body weight; FCR, feed conversion ratio.

3.4.3 Carnosine Content on Meat Quality and Lipid Oxidation

Amino acid supplementation did not affect $pH_{24\ h}$ ($P>0.05$), whereas but led to an increase in $pH_{45\ min}$, the ability to retain water in meat and a decrease in the shear force ($P<0.05$). The results, shown in Table 4.4, demonstrated that chickens fed an amino acid-supplemented diet (groups B, C, and D) tended to produce superior meat quality compared to that of the control group.

Table 3.4 Effect of amino acids supplementation on meat quality of KRCs.

Parameter	Treatment group[1]				SEM[2]	P-value
	A	B	C	D		
pH_{45min}	5.27[b]	5.41[a]	5.33[ab]	5.40[a]	0.03	0.01
pH_{24hr}	5.36	5.39	5.39	5.38	0.02	0.759
Drip loss (%)	13.64[a]	13.17[ab]	11.11[b]	12.61[ab]	0.53	0.02
Cooking loss (%)	25.83[a]	24.55[b]	24.55[b]	24.05[b]	0.27	0.002
Shear force (kg)	3.31[a]	3.50[a]	2.94[b]	2.81[b]	0.08	<0.001

Results were averaged from 25 chickens per treatment.

[a-c] Mean values with different superscripts in the same row indicate significantly different at P-value <0.05

[1]Treatment groups are A (control), B (supplemented with 1.0% β-alanine), C (supplemented with 0.5% L-histidine), and D (supplemented with 1.0% β-alanine + 0.5% L-histidine) respectively.

[2]SEM indicates standard error of mean

The superior meat quality may be due to a reduction in the decline in postmortem pH ($pH_{45\ min}$). When an animal dies, carnosine removes lactic acid and regulates Ca^{2+} discharge from the sarcoplasmic reticulum, reducing ATP consumption during postmortem glycolytic metabolism (Culbertson et al., 2010). As carnosine acts as a proton-sequestering molecule produced by anaerobic glycolysis (Boldyrev et al., 2013), it leads to a gradual decline in $pH_{45\ min}$ with an increase in the water holding capacity of the final meat product, affecting its functional properties (Kim et al., 2016). Moreover, carnosine can prevent oxidative protein modification by combining with reactive carbonyl species to inhibit protein carbonylation (Hipkiss et al., 2001). As a result, the meat from the amino acid-supplemented group had lower drip loss, shear force, and cooking loss.

TBARS (mg MDA/kg of tissue) was the lowest in group D ($P<0.05$, Figure 3.1), demonstrating the anti-oxidant characteristics of carnosine. Metal ions catalyze the formation of advanced lipid oxidation end-products (Negre-Salvayre et al., 2008). The high level of carnosine in meat acts as a strong inhibitor of lipid oxidation end-products (Boldyrev et al., 2013), leading to lower lipid oxidation in broilers (Cong et al., 2017b).

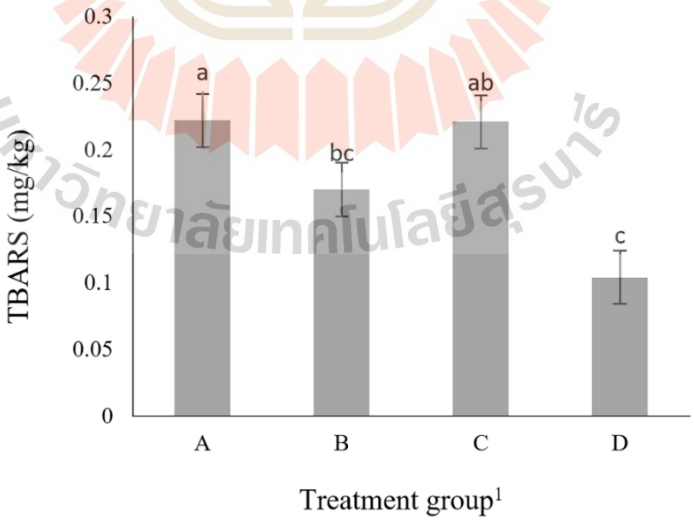

Figure 3.1 TBARS value in Korat chicken breast meat of the different experimental groups Results were averaged from 10 chickens per treatment.

3.4.4 SR-FTIR Application for Determining the Intensity Ratios of Biomolecules and Secondary Structure Proteins from Different Carnosine Contents

The average original and second derivative spectra in the fingerprint region of the wave number at 3000 to 900 cm^{-1} of KRC breast meat from the different amino acid supplementation groups are shown in Figures 3.2A and B, respectively. The average second derivative spectra from the 4 treatments clearly separated the peak high and peak ratios at 1664 cm^{-1}, 1650 cm^{-1}, and 1635 cm^{-1} representing amide I and 1587 cm^{-1}, 1550 cm^{-1}, and 1529 cm^{-1} representing amide II, respectively.

The percentages of integration of each biomolecule in Table 3.5 revealed significant differences between amides I and II ($P<0.05$). The integral area of amide I in group D was lower than that in the control group. In contrast, those of groups B and C were not significantly different compared with that of the control or group D. Regarding amide II, the integral areas of the control, B, and D groups were higher than that of group C ($P<0.05$). In addition to lipid, amide III, CH bending, and glycogen/carbohydrate showed non-significant differences ($P>0.05$). These results suggested that the supplemented amino acids strongly affected the change in the secondary structure of muscle proteins.

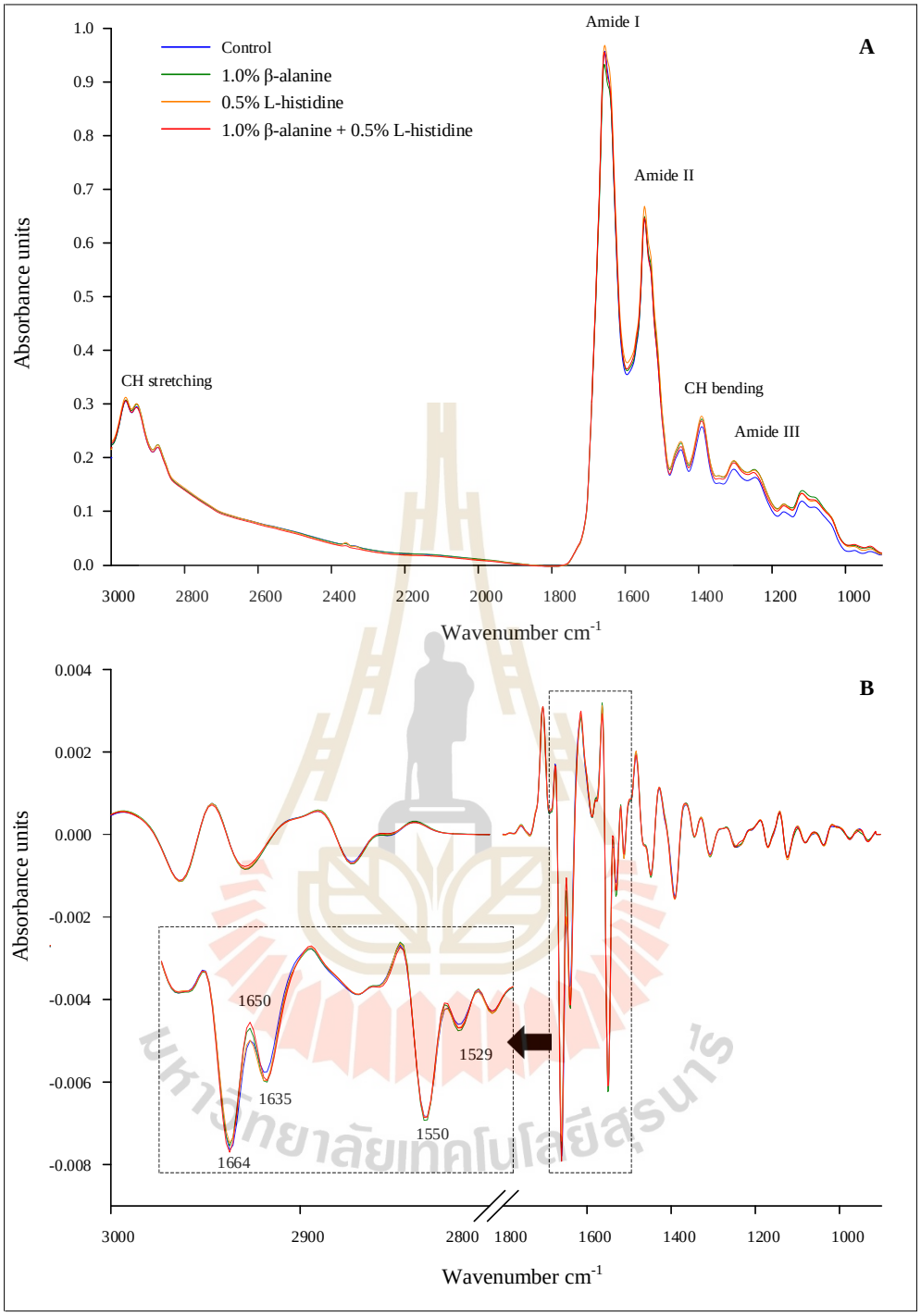

Figure 3.2 Average synchrotron radiation-infrared (SR-IR) spectra of the original spectra (a) and second derivative spectra (b) in the fingerprint region of the wave number at 3000 to 900 cm^{-1} in Korat chicken breast meat of the different experimental groups.

The secondary structure of the protein in the amide I region is composed of β-sheet, α-helix, β-turn, and antiparallel, and their integral areas are presented in Table 3.6. Significant differences in the secondary structures of amide I were detected. When the control group was used as the reference point, the relative β-sheet content in group D was lower than that of the control group, whereas the lowest relative β-turn content was found in the control group. These results confirm the results for amide I, shown in Table 3.5.

Table 3.5 The ratio of the integral area of biomolecules in KRCs breast meat determined using synchrotron radiation-Fourier transform infrared (SR-FTIR) microspectroscopy.

Biomolecule	**%Integral area**				**SEM**	**P-value**
(wavenumber)	**A**	**B**	**C**	**D**		
Lipid (3000-2800 cm^{-1})	10.49	10.69	11.04	10.67	0.29	0.610
Amide I (1700-1600 cm^{-1})	42.01^{a}	39.52^{ab}	40.03^{ab}	38.43^{b}	0.73	0.023
Amide II (1600-1550 cm^{-1})	29.68^{ab}	32.62^{a}	28.87^{b}	32.57^{a}	0.75	0.004
CH bending (1450-1390 cm^{-1})	5.54	5.81	6.79	5.95	0.41	0.198
Amide III (1320-1220 cm^{-1})	3.38	3.91	3.99	3.61	0.28	0.421
Glycogen/Carbohydrate (1200-900 cm^{-1})	7.98	6.92	8.12	8.23	0.35	0.065

Results were averaged from 400 spectra per treatment.

Treatment groups are A (control), B (supplementation with 1.0% β-alanine), C (supplementation with 0.5% L-histidine), and D (supplementation with 1.0% β-alanine and 0.5% L-histidine), respectively.

$^{a-b}$ Mean values with different superscripts in the same row indicate significantly different at P-value<0.05.

Table 3.6 Ratio of secondary structures in KRCs breast meat determined using synchrotron radiation-Fourier transform infrared (SR-FTIR) microspectroscopy.

Trait	% Curve fitting				SEM	P-value
	A	B	C	D		
β-sheet (1630 cm^{-1})	22.24^{a}	19.85^{b}	20.71^{ab}	18.72^{b}	0.536	0.002
α-helix (1644 cm^{-1}, 1655 cm^{-1})	49.44^{ab}	49.59^{ab}	47.85^{b}	50.74^{a}	0.596	0.028
β-turn (1670 cm^{-1})	15.61^{c}	19.09^{a}	18.14^{ab}	17.63^{b}	0.244	0.000
Antiparallel (1689 cm^{-1})	12.70^{b}	11.68^{b}	14.50^{a}	12.92^{b}	0.384	0.001

Results were averaged from 400 spectra per treatment.

Treatment groups are A (control), B (supplementation with 1.0% β-alanine), C (supplementation with 0.5% L-histidine), and D (supplementation with 1.0% β-alanine and 0.5% L-histidine), respectively.

$^{a-c}$ Mean values with different superscripts in the same row indicate significantly different at P-value<0.05.

The PCA score plot of the chicken meat biomolecule spectra is shown in Figure 3.3A, demonstrating that chicken meat was separated into 4 groups according to the experimental groups. The combination of PC1 and PC2 at 88% of the total variance could explain this finding. Figures 3.3B and C show the details of the biomolecules that differentiated the meat in different groups. When the findings shown in Figure 3.3A and C were considered together, it was apparent that groups B and D were positively correlated with β-sheet (wavenumber 1687 cm^{-1}, 1635 cm^{-1}, 1529 cm^{-1}, and 1255 cm^{-1}), β-turn (wavenumber 1664 cm^{-1}), doublet due to the 2 protonated tautomers of histidine (1587 cm^{-1}), amide II (1550 cm^{-1}), and CH and CH_2 aliphatic bending (wavenumber 1448 cm^{-1}) groups. Groups A and C were positively correlated with α-helix (wavenumber 1650 cm^{-1} and 1540 cm^{-1}), tyrosine (wavenumber 1519 cm^{-1}), and CH and CH_2 aliphatic bending groups (wavenumber 1457 cm^{-1} and 1365 cm^{-1}). The reference SR-FTIR bands with high correlation loading in KRC breast meat are shown in Table 3.7.

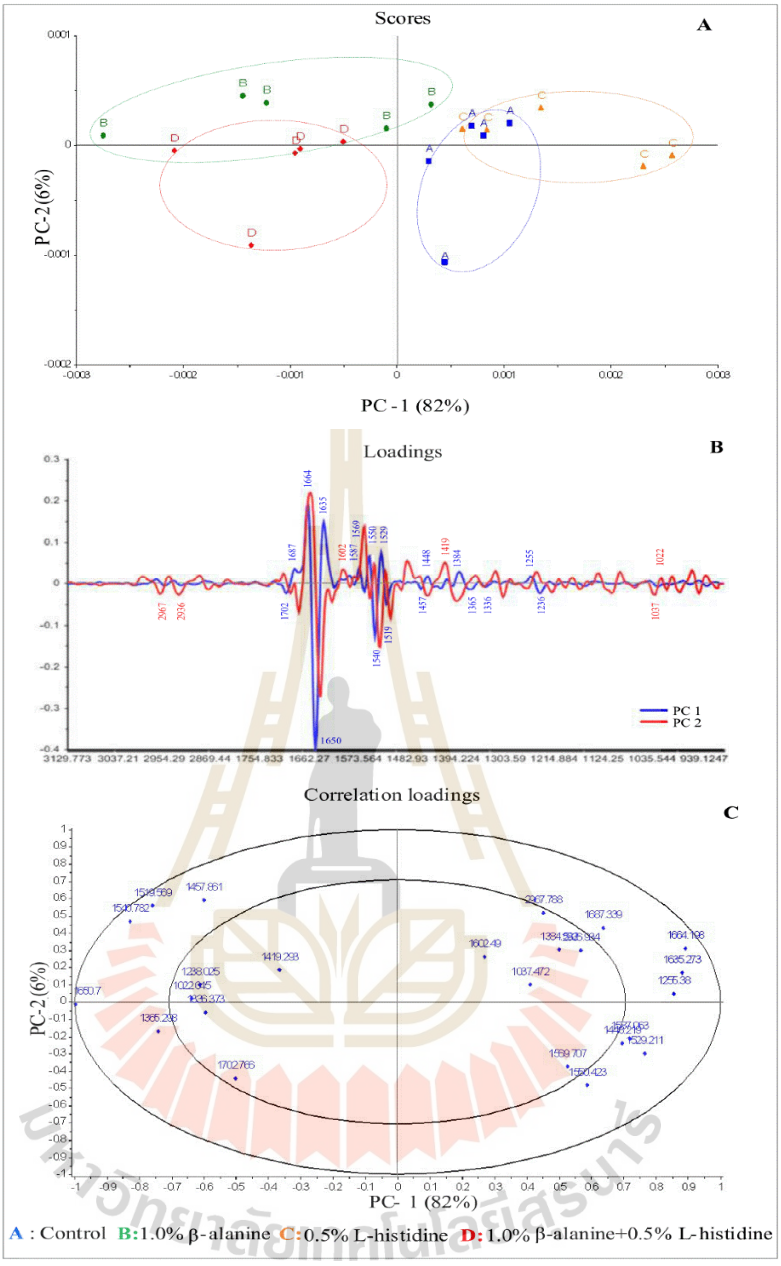

Figure 3.3 PCA score plot (a) for PC1 versus PC2 for 4 different experimental data, loading plot (b) detail of the effects of biomolecules on the meat of the different experimental groups, and correlation loading plot (c) for PC1 versus PC2 for biomolecules, at 88% total variance of Korat chicken breast meat of the different experimental groups. Spectra were collected (400 spectra per treatment) using second derivative processing at the spectral regions from 3000 to 900 cm^{-1} by the outer and inner ellipse representing 100% and 50% of the variance, respectively.

Table 3.7 The related Fourier transform infrared (FTIR) band assignment observed spectra in KRCs breast meat.

Wavenumber (cm^{-1}) in literature	Wavenumber (cm^{-1}) in our study	Definition of the spectral assignments*	References
2957–2953	2960–2874	CH_3 asymmetric stretching Lipids (mainly), proteins	Candoğan et al. (2020)
1700–1715	1702	C= O stretching Fatty acid	Mecozzi et al. (2012)
1682	1687	Amide I of β-sheet	Bocker et al. (2007)
1659	1664	Amide I of β-turn	
1653	1650	Amide I of α-helix	
1639	1635	Amide I of β-sheet	
1575,1594	1587	Doublet due to the 2 protonated tautomers of histidine	Barth (2007)
1567	1569	Amide II of β-sheet	Bocker et al. (2007)
1556	1550	Amide II	
1544	1540	Amide II of α-helix	
1527	1529	Amide II of β-sheet	
1518	1519	Tyrosine	De Meutter and Goormaghtigh (2021)
1460–1350	1457–1365	CH and CH_2 aliphatic bending group	Mecozzi and Sturchio (2017)
1256	1255	Amide III of β-sheet	Singh et al. (1993)
1237	1238		
1035	1037	C-O, C-C str., C-O-H, C-O-C def. (of carbohydrates)	Lazar et al. (2012)
1020–1022	1022	Glycogen	

*Resolution of 6 cm^{-1}

As demonstrated by these results, the carnosine content can affect the quality of the meat related to its texture and water retention ability, which is consistent with previous studies (Cong et al., 2017a; Kralik et al., 2018; Qi et al., 2018). However, this study is the first to use SR-FTIR to monitor biomolecules in meat with different carnosine contents, and the SR-FTIR results showed that different carnosine contents could change the relative contents of some biomolecules in meat. Correlation loading analysis using the physicochemical characteristics, integral area of the biomolecule, integral area of the secondary structure of proteins, and wave number from Figure 3.3C was performed to make these data more informative, and the results are discussed in the next subtopic.

3.4.5 Correlation Loadings Plot of PCA between SR-FTIR Spectra and Physicochemical Results from Different Carnosine Contents

The score plot and correlation loading that expresses the relationship between SR-FTIR spectra and physicochemical results are shown in Figure 3.4 (upper and lower parts, respectively). This relationship explained approximately 51% of the total variation. All variables located in the outer circle region (carnosine, $pH_{45\ min}$, amide II, cooking loss, TBARS, and wavenumber 1664 cm^{-1}, 1650 cm^{-1}, 1635 cm^{-1}, 1587 cm^{-1}, 1540 cm^{-1}, 1529 cm^{-1}, 1519 cm^{-1}, 1457 cm^{-1}, 1448 cm^{-1}, 1365 cm^{-1}, and 1255 cm^{-1}) showed a significant correlation with the 4 experimental groups with a variance greater than 50%. Group A (Figure 4, upper part) was positively correlated with TBARS, cooking loss, α-helix (wave number 1540 cm^{-1}), tyrosine (wave number 1519 cm^{-1}), and CH and CH_2 aliphatic bending groups (wave number 1457 cm^{-1}) (Figure 4, lower part). In group B, a positive correlation was found with amide II, β-sheet (wave numbers 1635 cm^{-1}, 1529 cm^{-1}, and 1255 cm^{-1}), β-turn (wave number 1664 cm^{-1}), and CH and CH_2 aliphatic bending groups (wave number 1448 cm^{-1}). Group C was positively correlated with α-helix (wave number 1650 cm^{-1}) and CH and CH_2 aliphatic bending groups (wave number 1365 cm^{-1}). In group D, a positive correlation was found with the carnosine content, $pH_{45\ min}$, and doublet due to the 2 protonated histidine tautomers (wave number 1587 cm^{-1}).

From the results, 2 interesting points need to be discussed. The first point is that carnosine levels can affect the physiological properties of meat by changing biomolecules in meat, as demonstrated using SR-FTIR. The second point is that β-

alanine or L-histidine supplementation may decrease meat quality because it affects the increase in cooking loss and levels of TBARS, as demonstrated by the results, which aligned with those shown in Table 3.4 and Figure 3.1, respectively.

Supplementation with both amino acids (group D) can increase $pH_{45\ min}$, leading to a better maintenance of $pH_{45\ min}$. Simultaneously, the TBARS value decreased, resulting in an improved water-holding ability of muscle fibers. Interestingly, supplementation with both amino acids also increased the integral area to 1587 cm^{-1}, representing the binding of carnosine to Cu^{2+} (Torreggiani et al., 2000). This form of carnosine can act as a chelator ion, improving the anti-oxidant capacity (Güner and Alpsoy, 2015). Cong et al. (2017a) reported that increased anti-oxidant levels could improve the ability to maintain $pH_{45\ min}$, decrease drip loss, cooking loss, and shear force. The results suggest that supplementation improves meat quality by changing the level of biomolecules in meat, as demonstrated by the SR-FTIR results. The effect of carnosine on anti-oxidant capacity needs to be investigated in future studies to confirm this.

Supplementation with either β-alanine or L-histidine has adverse effects on the physiological properties of meat, such as an increase in cooking loss and TBARS. The results may be caused by the increase in β-sheet, β-turn, and aliphatic bending groups. Residual amino acids from carnosine synthesis, free amino acids, might induce the oxidative modification of proteins (Zhang et al., 2013). Protein oxidation causes the unfolding of its secondary structure, which is transformed into β-sheets, β-turns (Li et al., 2020), and aliphatic bending groups (Herrero, 2008). Katemala et al. (2021) reported that the β-sheet relative content positively correlated with the shear force of KRC, and the result was confirmed by Beattie et al. (2004). Moreover, Kubota et al. (2021) found that the upregulated genes *LOC107051274*, *ACSBG1*, and *CAPNS2* and downregulated genes *MYO7B*, *MYBPH*, *SERPINH1*, and *PGAM1* may be related to meat tenderness in Korat chicken. However, it is still unclear how these genes change the protein secondary structure in this study. Further studies are required to clarify the molecular mechanism involved in meat quality.

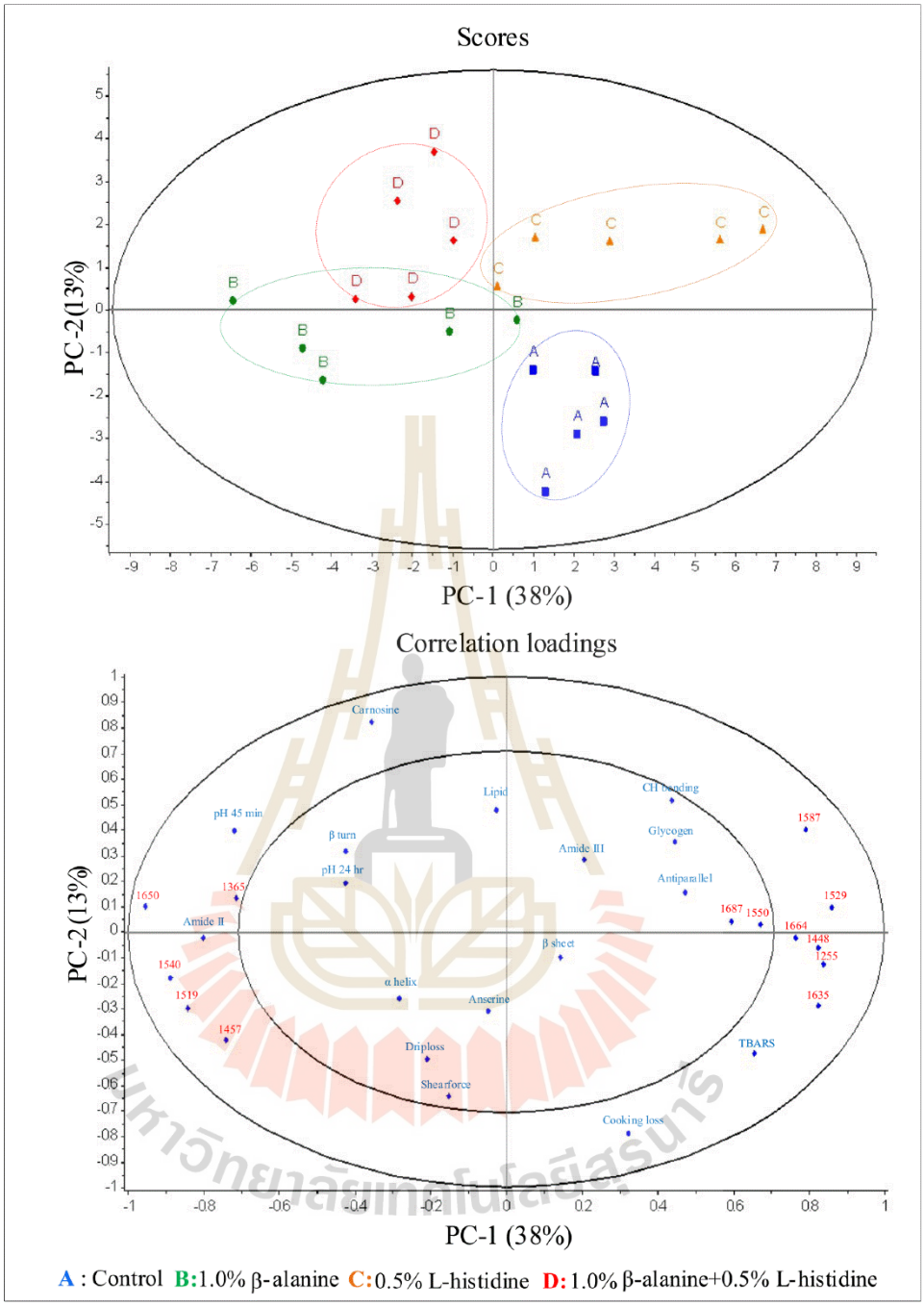

Figure 3.4 PCA score plot (upper) for PC1 versus PC2 for the 4 different experimental data and correlation loading plot (lower) for PC1 versus PC2 for physicochemical properties, biomolecules, secondary structure protein at 88% total variance in Korat chicken breast meat of the different experimental groups by the outer and inner ellipse representing 100% and 50% of the variance, respectively.

3.5 Conclusions

This study confirmed that the carnosine content could be increased by amino acid supplementation (substrates of carnosine synthesis) in slow-growing KRC meat. Supplementation cannot improve the performance of chickens because of the ability of carnosine to resist oxidative stress. Furthermore, the highest carnosine synthesis was observed with supplementation of both β-alanine and L-histidine. Moreover, the water retention ability of muscle cells and $pH_{45\ min}$ can be improved. In addition, the results suggest that single β-alanine or L-histidine supplementation may negatively affect physiological properties, confirmed by the increase in β-sheet, β-turn, and aliphatic bending groups. However, we used only one level of both amino acids. Further studies are required to examine the optimum level of supplemented amino acids and genetics involved with carnosine synthesis.

3.6 Acknowledgments

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CHAPTER IV

REVEALING THE GLOBAL MECHANISM RELATED TO CARNOSINE SYNTHESIS IN THE PECTORALIS MAJOR OF THE SLOW-GROWING KORAT CHICKEN USING A PROTEOMIC APPROACH

4.1 Abstract

This study aimed to find global molecular mechanisms and cellular functions related to carnosine synthesis in the slow-growing Korat chickens (**KRC**) using a proteomic approach. *M. pectoralis major* samples were collected from 10-week-old female KRC, and they were separated into two different carnosine content groups: Low-carnosine (fed with basal diet; n=5) and High-carnosine (fed with the basal diet supplemented with 1.0% β-alanine+0.5% L-histidine; n=5). Using a label-free quantitative proteomic approach**,** we identified 399 proteins, and 8 of these proteins showed differential expression between the 2 groups ($P<0.05$). Using function enrichment, Kyoto Encyclopedia of Genes and Genomes pathway, and protein–protein network analyses, the proteins Heat shock 70 kDa protein 8 (**HSPA8**), heat shock 70 kDa protein 2 (**HSPA2**), Protein disulfide isomerase family A, member 6 and Endoplasmic reticulum resident protein 29 were significantly enriched in protein processing in the endoplasmic reticulum pathway (false discovery rate, FDR<0.05). The results suggested that the pathway is related to carnosine synthesis in the *M. pectoralis major* of the slow-growing KRC. Carnosine is mainly involved in stimulating Titin isoform Ch12 (**TTN**), Connectin, and M-protein (**MYOM2**) expressions to maintain homeostasis and to regulate stress in skeletal and cardiac muscle cells that finally results in the desired meat characteristics. Principal component analysis confirmed this, in which carnosine had positive loadings for Lumican, Fatty acid binding protein 3, HSPA8, HSPA2, MYOM2, β-turn, α-helix, and $pH_{45\ min}$, and had negative loadings for TTN, Connectin, β-sheet, drip loss, cooking loss, shear force, and thiobarbituric acid reactive substances. Thus, the carnosine synthesis may occur when cells need to recover homeostasis, leading to improved muscle function with no adverse effects

on health or meat characteristics. To the best of our knowledge, this is the first study to describe in detail the global pathways for carnosine synthesis, its role in cellular function, and the consequences of its synthesis and function based on the proteomic approach.

Keywords: carnosine synthesis, molecular pathway, proteomic approach, slow-growing chicken

4.2 Introduction

Slow-growing Korat chicken (**KRC**) is a crossbred chicken between the Thai indigenous sire and Suranaree University of Technology synthetic breed dam. KRC meat has predominant α-helices and contains less fat, low purine, and higher quantities of protein than commercial broilers (Katemala et al., 2021). Developing nutrient-rich chicken meat, which is fast-growing in the healthy-food industry, has increased the competition with other healthy products (Pinto et al., 2021).

Carnosine is a naturally occurring dipeptide histidine present in the body (Hipkiss et al., 2013) and is synthesized from a combination of β-alanine and L-histidine using the enzyme carnosine synthase (Boldyrev et al., 2013). Carnosine is beneficial for human health (Xing et al., 2021) as it can suspend biochemical processes via scavenging free radicals, chelating metals, and maintaining buffer capacity (Güner and Alpsoy, 2015). Interestingly, carnosine is mainly found in muscle and brain tissue of vertebrates or mammals (Everaert et al., 2011). Previous studies (Mateescu et al., 2012; D'Astous-Pagé et al., 2017) have reported that the effect of genes is one of the sources of variation in carnosine content in animals. Therefore, our breeding goals was to include genetically improved KRC as a source of additional carnosine. Furthermore, for genetic improvement, the holistic pathways related to carnosine synthesis and function and their consequences need to be understood in detail to precisely design breeding strategies and gene markers to avoid undesirable impact on animal health and their meat, among others.

The molecular processes involved in carnosine synthesis were first described by Cong et al. (2017), Qi et al. (2018), and Qi et al. (2021) in fast-growing chickens. They found that carnosine synthesis was related to the expression of the genes

histidine decarboxylase (***HDC***), proton-coupled oligopeptide transporters (***PEPT1***), solute carrier family 6 member 6 (***SLC6A6***), and carnosine synthase (***CARNS***). Additionally, Khumpeerawat et al. (2021) and Sharma et al. (2022) found that the genes carnosine synthase1 (***CARNS1***) and solute carrier family 36, member 1 (***SLC36A1***) transporter were involved in carnosine synthesis in slow-growing chickens. Moreover, Drozak et al. (2010) had reported that the ATP-grasp domain-containing protein 1 (**ATPGD1**) was related to carnosine synthesis. All the above-mentioned genes and proteins are specific to carnosine synthesis; however, the holistic pathways, genes or proteins involved in carnosine synthesis, carnosine function, and its consequences still need to be understood.

Recently, our group reported that the increase in carnosine content in meat can decrease pH ($pH_{45\ min}$) and the value of thiobarbituric acid reactive substances (**TBARS**) that consequently prevents a change in the protein secondary structure, resulting in improved water-holding ability in the breast meat of KRC (Suwanvichanee et al., 2022). These results align with that obtained by Cong et al. (2017), Qi et al. (2018), and Baldi et al. (2021), which suggest that an increase in carnosine content in the *M. pectoralis major* muscle of chicken plays a role in regulating postmortem pH decline that induces muscle metabolism during pre-rigor phase and consequently improves meat characteristics.

Transcriptomics or proteomics is a powerful approach to reveal global pathways. Till date, only one study by Kubota et al. (2021) has described the transcriptomic profile related to carnosine synthesis in the breast meat of female KRC fed with L-histidine or β-alanine as dietary supplements. Pathways associated with meat tenderness and oxidative stress resistance were discovered but that of carnosine synthesis were not found. However, Suwanvichanee et al. (2022) found that supplementing with β-alanine and L-histidine produced the highest carnosine content. Thus, both amino acids were considered limiting factors for carnosine synthesis in chickens (Brosnan and Brosnan, 2020).

The current study utilizes a proteomic approach as proteins and enzymatic biological functions mostly determine phenotypic diversity from a set of common genes (Rifai and Gerszten, 2006).

Hence, to the best of our knowledge, we describe for the first time the pathways related to carnosine synthesis, its molecular functions, and its role in muscle functions and structure using proteomics, focusing on *M. pectoralis major* tissue in the slow-growing KRC.

4.3 Materials and methods

4.3.1 Ethics Statement

All animals used adhered to the guidelines approved and provided by the Ethics Committee on Animal Use of Suranaree University of Technology, Nakhon Ratchasima, Thailand; document ID: U1-02631-2559.

4.3.2 Animals and Sample selection

Ten *M. pectoralis major* samples from female KRC were selected based on the significant difference in carnosine content from our previous study (Suwanvichanee et al., 2022). For this study, ten female KRC breast meat samples were separated into 2 groups: Low-carnosine (**LC**; n=5) and High-carnosine (**HC**; n=5). Briefly, chickens in LC and HC groups were fed with basal diet and basal diet supplemented with 1.0% β-alanine (Sigma-Aldrich, St. Louis, MO, USA, 146064) + 0.5% L-histidine (AppliChem GmbH, Darmstadt, Germany, A3738), respectively. At 10 weeks of age, the chickens were slaughtered and *M. pectoralis major* samples were collected and meat characteristics and carnosine content were measured. A detailed description of the formulation diets is shown in Table 1.

For proteomic analysis, breast meat tissue of approximately 10 g from each sample was collected from similar parts of the breast used for the carnosine content measurement. They were then snap-frozen in liquid nitrogen and kept frozen at -80°C until used for protein extraction.

Table 4.1 Ingredient and nutrient composition of experimental diets in different growing phases.

	Grower (22 to 42 days)[1]		Finisher (43 to 70 days)[1]	
	A	B	A	B
Ingredients (kg)				
Corn	59.40	56.64	67.30	64.44
Soybean meal (44% CP)	30.80	28.78	26.30	26.70
Full fat soybean	2.42	5.20	0.00	0.00
Rice bran oil	4.00	4.50	3.04	4.00
L-Lysine	0.18	0.18	0.19	0.19
DL-Methionine	0.21	0.21	0.14	0.14
Salt	0.35	0.35	0.35	0.35
Calcium carbonate	1.42	1.42	1.20	1.20
MDCP (P21)	1.02	1.02	1.28	1.28
Premix[2]	0.20	0.20	0.20	0.20
β-Alanine	0	1.00	0	1.00
L-Histidine	0	0.50	0	0.50
Total (kg)	100	100	100	100
Calculated composition (%)				
ME (kcal/kg)	3,113	3,111	3,112	3,110
Crude protein, %	19.39	19.29	17.08	17.04
Crude fiber, %	3.60	3.55	3.32	3.29
Ether extract, %	6.94	7.82	5.81	6.66
Calcium, %	0.90	0.90	0.86	0.86
Total phosphorus, %	0.57	0.56	0.60	0.59
Histidine	0.45	0.98	0.37	0.87

[1]Treatment groups are A (Low-carnosine group) and B (High-carnosine group)

[2]Premix (0.5%) provided the following per kilogram of diet: 15,000 IU of vitamin A, 3,000 IU of vitamin D3, 25 IU of vitamin E, 5 mg of vitamin K3, 2 mg of vitamin B1, 7 mg of vitamin B2, 4 mg of vitamin B6, 25 ug of vitamin B12, 11.04 mg of pantothenic acid, 35 mg of nicotinic acid; 1 mg of folic acid, 15 µg of biotin, 250 mg of choline chloride, 1.6 mg of Cu, 60 mg of Mn, 45 mg of Zn, 80 mg of Fe, 0.4 mg of I and 0.15 mg of Se.

4.3.3 Protein extraction

Proteins were extracted according to a previously described method (Kaewsatuan et al., 2022). Breast meat samples were freeze-dried using the freeze dryer GAMMA 2-16 LSCplus (Martin Christ, Gefriertrocknungsanlagen GmbH, Osterode, Germany) and finely ground to a powder. Breast powder was ice-sonicated in 50 mM ammonium bicarbonate buffer (**AMBIC**) containing 8 M urea (Sigma-Aldrich, USA). The extract proteins were centrifuged at 20,000×g for 10 min at 4°C. The supernatants were collected and diluted with 50 mM ammonium bicarbonate buffer to a final concentration of 1.5 M urea. Then, the concentrations of protein were measured using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, USA).

From each supernatant sample, 100 µg of protein was reduced to disulfide bonds by adding dithiothreitol (**DTT**) to a final concentration of 5 mM and incubated between 50 and 60°C for 20 min. Then, iodoacetamide (**IAA**) was added to a final concentration of 15 mM and was incubated in the dark at 25°C for 20 min. Subsequently, 2 µg of trypsin (Promega, USA) was added and kept overnight at 37°C to digest the protein sample into peptides.

4.3.4 Mass spectrometry

Each protein sample (4 µg) was desalted with C18 columns and separately analyzed with a TripleTOF® 6600 Quadrupole Time-Of-Flight instrument (Sciex, USA) coupled to an Eksigent nanoLC (Eksigent Technologies, Dublin, CA, USA) with a Turbo V Mass spectrometry analysis *performed in the* microspray mode at the Proteomics Unit core facility, University of Helsinki, Finland. YMC-Triart C18 column (12 nm, 3 µm, 150 x 0.3 mm) (YMC CO, Japan) was used for peptide separation. The MS-data-independent acquisition (**DIA**) in positive ion mode, using a linear 60-min gradient from 5 to 35 % buffer B (0.1% formic acid in acetonitrile). An *accumulation time* of 250 ms was used for survey scans acquired and the top 30 ions above the intensity threshold of 150 counts were selected for subsequent MS/MS scans (100 to1500 m/z, 100 ms accumulation time per MS/MS). The raw proteomic data sets in the current study are deposited in ProteomeXchange Consortium via the PRIDE (https://www.ebi.ac.uk/pride/) partner repository with the data set PXD038020 (Reviewer account details: Username: reviewer_pxd038020@ebi.ac.uk; Password: RtqJ0A9s).

4.3.5 Protein identification

The raw .wiff files were obtained using MaxQuant software version 1.6.5.0 (Cox and Mann, 2008) for protein identification. Peptides were identified using UniProtKB *Gallus gallus* database (total 34,827 entries, downloaded in March 2022 from https://www.uniprot.org). The parameters of protein identification were set as trypsin specificity enzyme with 2 missed cleavages. Carbamidomethylation of cysteine residues and oxidation of methionine was used as static modification and dynamic modification, respectively. The first search and main search were set to 0.07 and 0.006 Da, respectively. The fragment (MS/MS) mass deviation was set to 20 ppm; peptide and protein false discovery rate (**FDR**) were both set at 1%. Match-between-runs algorithm was used with a match time window of 0.7 min and an alignment time search space of 20 min. Razor and unique peptide were used for label-free quantification (**LFQ**) algorithm (minimum ratio count = 2).

4.3.6 Differential proteomic analyses

Statistical analyses and data visualization was performed in Perseus software version 1.6.5.0 (Tyanova et al., 2016). LFQ intensity data generated from Maxquant were loaded into Perseus. The data was filtered by removing protein identified with post-translation modification, contaminant proteins, or hits related to the reverse sequence. For quantification, proteins were kept if they appeared in at least 2 out of 5 biological replicates of both experimental groups. The data was transformed to a logarithmic scale with base 2. Missing values were then imputed. For the comparison between LC and HC groups, Student's t-test was used with $P < 0.05$ considered as statistically significant differential abundant proteins (**DAPs**). Hierarchical clustering was then conducted with DAPs after Z-score normalization.

4.3.7 Bioinformatics analysis

The lists of DAPs and exclusive proteins of LC and HC groups were combined for functional enrichment analysis. The significant difference in the enriched Gene Ontology (**GO**) terms composed of Biological Process (**BP**), Cellular Component (**CC**), Molecular Function (**MF**), and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathway was considered at *P* adjusted by FDR <0.05 (Benjamini & Hochberg, 1995). The interactions between protein and protein networks (**PPI**) of DAPs and exclusive proteins in each group were identified using STRING version 10

(http://string-db.org) against the database of *Gallus gallus*, and the interactions were considered at a medium confidence score of 0.4 (Szklarczyk et al., 2015).

4.3.8 Principal component analysis

Principal component analysis (**PCA**) was applied for clustering and investigating the relationship between parameters of meat characteristics (data from our previous study by Suwanvichanee et al. (2022)), including carnosine content, meat characteristic traits ($pH_{45\ min}$, $pH_{24\ h}$, drip loss, cooking loss, and shear force), thiobarbituric acid reactive substances (**TBARS**) value, and secondary structure ratio (α-helix, β-sheet, β-turn), and DAPs intensity using Unscrambler® X Multivariate Data Analysis software (version 10.1, Camo Analytics, Oslo, Norway). The related variables among the data were represented by a correlation loading plot. All the variable data were weighted using a standard deviation weighting process before the analysis using PCA bi-plot correlation.

4.4 Results

4.4.1 Analysis of differential proteins involved in carnosine synthesis

A total of 399 proteins were identified in the *M. pectoralis major* of the LC and HC groups. The results from the mass spectrometry analysis are summarized in Tables S2 and S3. A total of 166 and 163 proteins were detected in the LC and HC groups, respectively, as shown using a Venn diagram in Figure 1. Fourteen and 11 proteins were exclusively identified in the LC and HC groups, respectively, and the respective protein names are shown in Tables 2 and 3. Among the 152 common proteins, 8 proteins were significantly different in abundance ($P<0.05$).

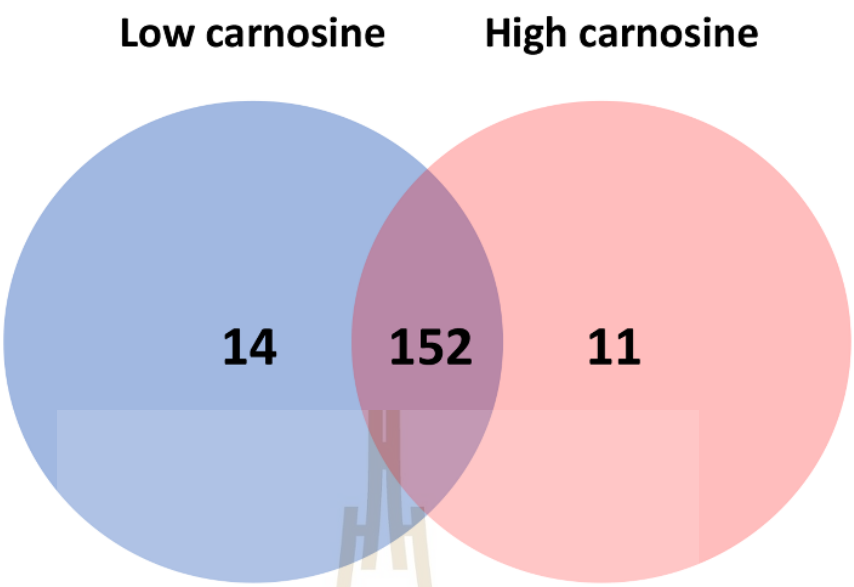

Figure 4.1 Venn Diagram representing the number of common proteins (center) and exclusive proteins identified in the KRC breast meat in Low- (left) and High-carnosine content (right).

Table 4.2 The list of proteins exclusively identified in the Low-carnosine group.

Protein ID	Protein name	Gene name
A0A1D5P470	Glutathione S-transferase	*GSTT1*
A0A1D5P893	Poly(rc)-binding protein 2 isoform	*PCBP2*
A0A1D5PDV6	Ribosomal protein S19	*RPS19*
A0A1D5PFA5	Myosin, light chain 10, regulatory	*MYL10*
P15989	Collagen alpha-3(VI) chain	*COL6A3*
A0A1D5PMT8	60S acidic ribosomal protein P2	*RPLP2*
A0A1D5PN46	Fructose-bisphosphatase	*FBP2*
A0A1D5PPF8	Heat shock 70kDa protein 4	*HSPA4*
A0A1D5PG30	Elongation factor 1-delta isoform	*EEF1D*
F1NHW5	Cytochrome b-c1 complex subunit 6	*UQCRH*
E1BVU4	SH3 domain-binding glutamic acid-rich-like protein	*SH3BGR*
F1NCA2	Glycerol-3-phosphate dehydrogenase	*GPD2*
F1NY09	Uncharacterized protein	*C1H11ORF54*
P07630	Carbonic anhydrase 2	*CA2*

Table 4.3 The list of proteins exclusively identified in the High-carnosine group**.**

Protein ID	Protein name	Gene name
A0A1D5P470	Glutathione S-transferase	*GSTT1*
A0A1D5P893	Poly(rc)-binding protein 2 isoform	*PCBP2*
A0A1D5PDV6	Ribosomal protein S19	*RPS19*
A0A1D5PFA5	Myosin, light chain 10, regulatory	*MYL10*
P15989	Collagen alpha-3(VI) chain	*COL6A3*
A0A1D5PMT8	60S acidic ribosomal protein P2	*RPLP2*
A0A1D5PN46	Fructose-bisphosphatase	*FBP2*
A0A1D5PPF8	Heat shock 70kDa protein 4	*HSPA4*
A0A1D5PG30	Elongation factor 1-delta isoform	*EEF1D*
F1NHW5	Cytochrome b-c1 complex subunit 6	*UQCRH*
E1BVU4	SH3 domain-binding glutamic acid-rich-like protein	*SH3BGR*
F1NCA2	Glycerol-3-phosphate dehydrogenase	*GPD2*
F1NY09	Uncharacterized protein	*C1H11ORF54*
P07630	Carbonic anhydrase 2	*CA2*

The hierarchical clustering of 8 DAPs showed a clear separation between the LC and HC groups (Figure 2), and the proteins were Heat shock 70 kDa protein 8 (**HSPA8**), Titin isoform Ch12 (**TTN**), Connectin, M-protein (**MYOM2**), Fatty acid binding protein 3 (**FABP3**), Lumican (**LUM**), Histone H4 (**H4-VIII**) and heat shock 70 kDa protein 2 (**HSPA2**), as shown in Table 4. The results suggested that the identified proteins were involved in proteome homeostasis, heat stress, and muscle contraction.

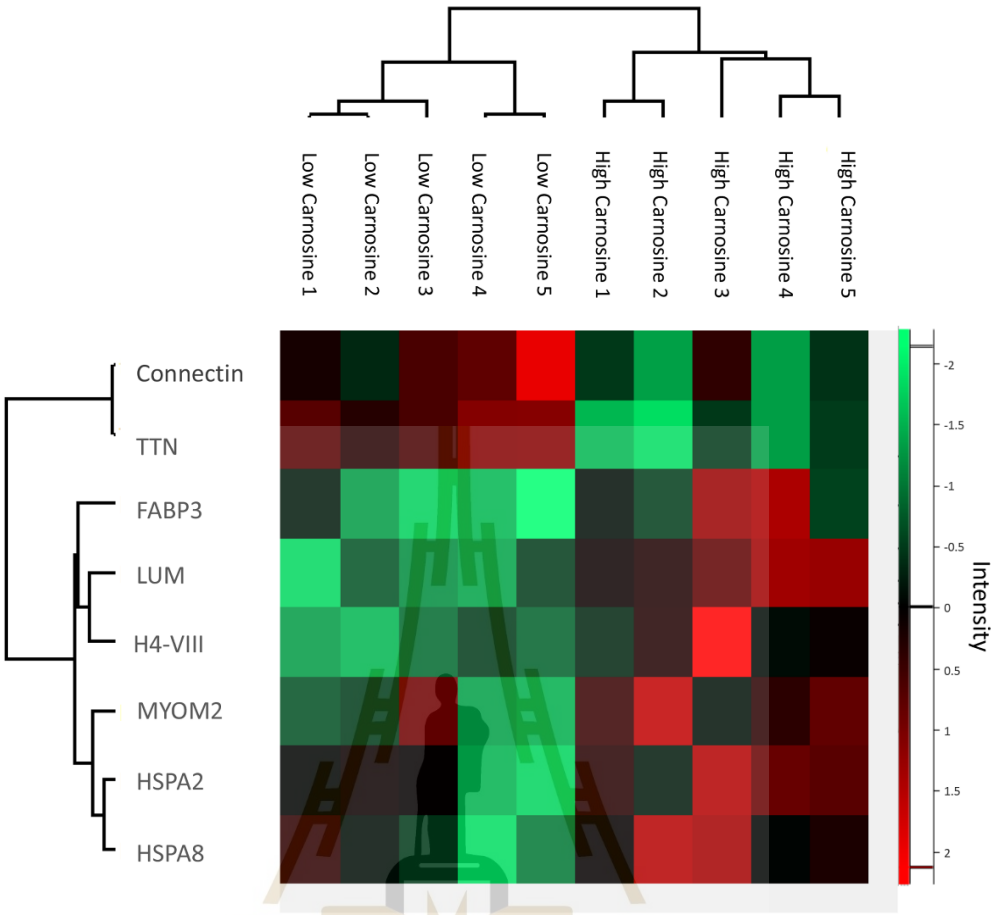

Figure 4.2 The predicted protein–protein interaction network from the combined protein of DAPs and exclusively of each group. Nodes represent the proteins from the *Gallus gallus* database and lines represent the connections between the proteins (green line–neighborhood; red line–fusion; blue line–co-occurrence; yellow–text mining; black–co-expression; and purple–protein homology) Abbreviations: DAPs, differential abundant protein.

Table 4.4 The differential abundant proteins (DAPs) in the KRC breast meat between Low- and High-carnosine content groups.

Uniport ID	Gene	Protein names	*P*-value	FC
A0A1D5PFJ6	*HSPA8*	Heat shock 70 kDa protein 8	0.042	-1.233
A6BLM7	*TTN*	Titin isoform Ch12 (Fragment)	0.000	1.903
A6BM71	*TTN*	Connectin	0.034	1.272
Q02173	*MYOM2*	M-protein	0.045	-1.222
Q6DRR5	*FABP3*	Fatty acid binding protein 3	0.015	-1.719
P51890	*LUM*	Lumican	0.001	-1.850
P70081	*H4-VIII*	Histone H4	0.021	-1.439
B3VHV2	*HSPA2*	Heat shock 70kDa protein 2	0.035	-1.269

Abbreviations: FC: fold change

4.4.2 Association of different proteins and their biological and physical parameters

PCA results showed that the HC group clearly differed from the LC group at a variance greater than 50% identified by the outer and inner ellipse that represents 100% and 50% of the variance, respectively (Figure 3, A). Carnosine content; LUM, FABP3, HSPA8, HSPA2, and MYOM2; β-turn; α-helix; and $pH_{45\ min}$ positively correlated with each other, whereas they negatively correlated with TTN, Connectin, β-sheet, drip loss, cooking loss, shear force, and the TBARS value (Figure 3, B). PCA results confirmed the comparative proteomic results.

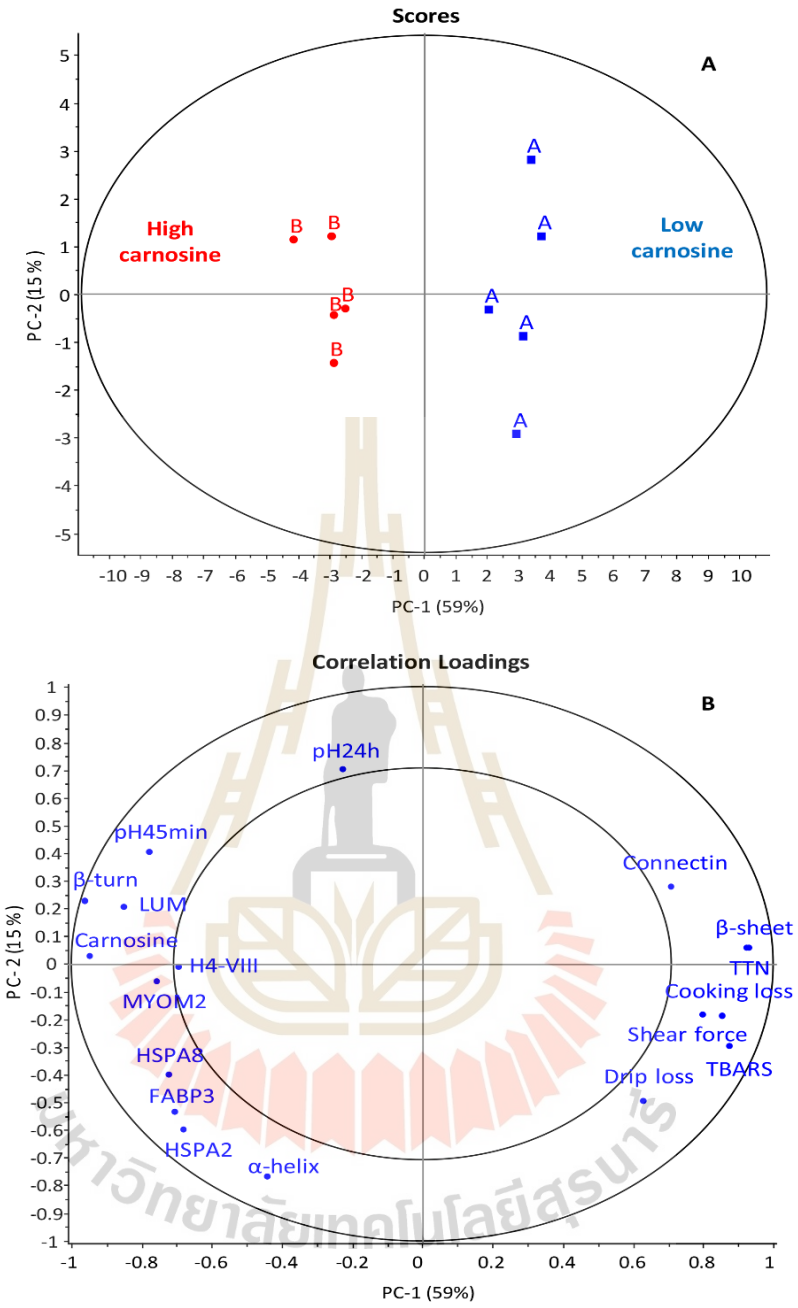

Figure 4.3 PCA (A) and correlation loading plot (B) for 8 DAPs, carnosine content, meat characteristics, and the change of protein secondary structure at 74% total variance for PC1 versus PC2 in Korat chicken breast meat of A, Low-carnosine and B, High-carnosine as indicated by the outer and inner ellipse, representing 100% and 50% of the variance, respectively. Abbreviation: PCA, principal component analysis; DAPs, differential abundant proteins.

4.4.3 Function enrichment analysis

The exclusive proteins from LC and HC groups and DAPs from the common group were combined to perform functional enrichment analysis to better understand the holistic mechanisms that are responsible for carnosine synthesis (Table 5).

The KEGG pathway analysis revealed that the common DAPs, HSPA8 and HSPA2, and the exclusive proteins in the HC group, Endoplasmic reticulum resident protein 29 (**ERP29**) and Protein disulfide isomerase family A, member 6 (**PDIA6**), were significantly enriched in protein processing in the endoplasmic reticulum pathway (FDR<0.05).

We also found the most significantly enriched GO terms that belonged to muscle contraction, protein muscle stability, and protein folding in the biological process category, such as the skeletal muscle thin filament assembly, skeletal muscle myosin thick filament assembly, or cardiac muscle fiber development. The significantly enriched terms for molecular function were composed of structural constituent of muscle and muscle alpha-actinin binding. The proteins involved in cellular components were mainly enriched in muscle fiber composed of striated muscle thin filament, actin cytoskeleton, Z-disc, and M-band.

Table 4.5 Functional enrichment analysis of differential abundant proteins (DAPs) and proteins exclusively identified in the Low- and High-carnosine groups.

Term ID	Term description	adj. *P* value[1]	Proteins
KEGG pathway			
gga04141	Protein processing in endoplasmic reticulum	0.034	HSPA8,HSPA2,**PDIA6,ERP29**
Biological process			
GO:0030240	Skeletal muscle thin filament assembly	0.007	TTN, Connectin
GO:0030241	Skeletal muscle myosin thick filament assembly	0.007	TTN, Connectin, MYOM2
GO:0048739	Cardiac muscle fiber development	0.007	TTN, Connectin
GO:0055003	Cardiac myofibril assembly	0.007	TTN, Connectin, MYOM2
GO:0045214	Sarcomere organization	0.027	TTN, Connectin, MYOM2
GO:0055008	Cardiac muscle tissue morphogenesis	0.038	TTN, Connectin, MYOM2
GO:0048769	Sarcomerogenesis	0.039	TTN, Connectin, MYOM2
GO:0035995	Detection of muscle stretch	0.047	TTN, Connectin, MYOM2
GO:0043933	Protein-containing complex subunit organization	0.047	TTN, Connectin, MYOM2, , **MIF, TTR**, H4-VIII, , HSPA8
Molecular function			
GO:0008307	Structural constituent of muscle	0.035	TTN, Connectin, MYOM2
GO:0051371	Muscle alpha-actinin binding	0.027	TTN, Connectin, MYOM2
Cellular component			
GO:0005865	Striated muscle thin filament	0.021	TTN, Connectin, MYOM2
GO:0015629	Actin cytoskeleton	0.021	TTN, Connectin, MYOM2
GO:0030018	Z disc	0.021	TTN, Connectin, MYOM2, **SYNPO**, **MYH7B**,
GO:0031430	M band	0.021	TTN, Connectin, MYOM2, **SYNPO**

[1] FDR-adjusted *P* value<0.05 (Benjamini, & Hochberg, 1995)

Bold character: exclusive protein in the High-carnosine group; red character; exclusive protein in the Low-carnosine group

4.4.4 Protein–Protein Interaction Network

The results of PPI using the STRING platform and 33 proteins are shown in Figure 4. Two separated networks (PPI enrichment at *P* value=1.55e-0.5) were revealed, and proteins related to stress-responsiveness (H4-VIII, HSPA8, HSPA2, Heat shock 70kDa protein 4 (**HSPA4**), Glutathione S-transferase (**GSTT1**), 60S acidic ribosomal protein P2 (**RPLP2**), and Elongation factor 1-delta isoform (**EEF1D**)) had a pivotal role in the network. The other set of proteins were related to cytoskeleton proteins (TTN, Connectin, Myosin, heavy chain 7B (**MYH7B**), and Myosin, light chain 10, regulatory (**MYL10**)) and ATP production (Fructose-bisphosphatase (**FBP2**), Glycerol-3-phosphate dehydrogenase (**GPD2**), and Cytochrome b-c1 complex subunit 6 (**UQCRHL**)). Moreover, 3 protein and 2 protein interactions were also found, comprising the connective tissue proteins (LUM, Polymerase I and transcript release factor (**PTRF**), and Collagen alpha-3(VI) chain (**COL6A3**)), endoplasmic reticulum proteins (ERP29 and PDIA6), and binding proteins (Transthyretin (**TTR**) and Fatty acid-binding protein, liver (**LBFABP**)). Additionally, we found independent proteins, composed of FABP3, UMP-CMP kinase (**CMPK1**), Endothelial differentiation-related factor 1 (**EDF1**), Synaptopodin (**SYNPO**), Macrophage migration inhibitory factor (**MIF**), O-acetyl-adp-ribose deacetylase 1 (**OARD1**), Poly(rc)-binding protein 2 isoform (**PCBP2**), Ribosomal protein S19 (**RPS19**), SH3 domain-binding glutamic acid-rich-like protein (**SH3BGR**), Uncharacterized protein (**C1H11ORF54**), and Carbonic anhydrase 2 (**CA2**). The results showed that carnosine synthesis and its functions were regulated by the reactions of the protein complex network.

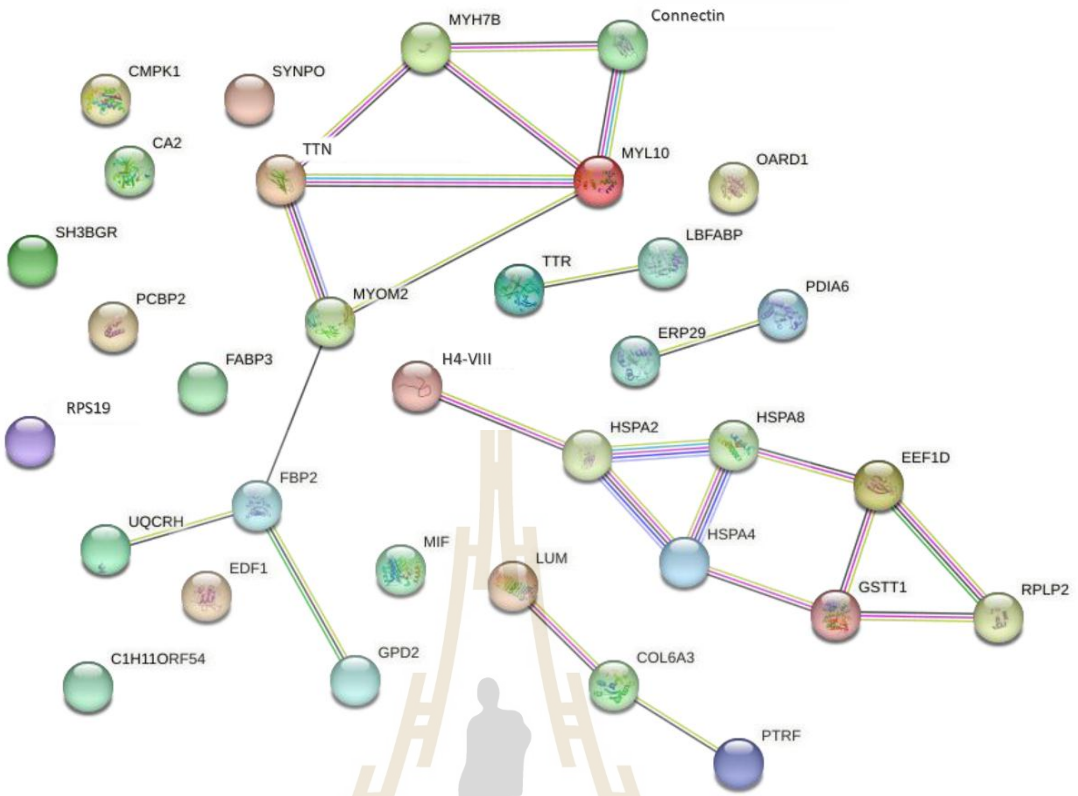

Figure 4.4 The predicted protein–protein interaction network from the combined protein of DAPs and exclusively of each group. Nodes represent the proteins from the *Gallus gallus* database and lines represent the connections between the proteins (green line–neighborhood; red line–fusion; blue line–co-occurrence; yellow–text mining; black–co-expression; and purple–protein homology) Abbreviations: DAPs, differential abundant protein.

4.5 Discussion

In this study, we aimed to describe that the global pathways related to carnosine synthesis, molecular functions, and their consequences on muscle structure and functions.

ATP-grasp Domain-containing Protein 1 (**ATPGD1**) related to carnosine synthesis (Drozak et al., 2010) was not found in our study and this might be due to the age of the chicken because the activity of the antioxidant enzymes diminishes as age increases (Rybka et al., 2011). This fact is supported by the findings of

Khumpeerawat et al. (2021), who found that *ATPGD1* expression linearly decreased when chicken age increases. The age of the chickens used in the current study (70-day-old) might be old to detect ATPGD1 expression. However, the results of our study describes for the first time the pathway and proteins associated with carnosine synthesis, cellular functions, and its consequences.

Interestingly, ATPGD1 and the pathways related to this protein may not be the only protein or pathway involved in carnosine synthesis, but the proteins found in this study (i.e., the protein processed in the endoplasmic reticulum (**ER**) and HSPA8, HSPA2, PDIA6, and ERP29) may also be associated with carnosine synthesis. The pathway of protein processed in the ER was revealed in the HC group that plays a role in glycosylation (Wang et al., 2015), involving the accurate folding of proteins and resisting the action of digestive enzymes (Phillips et al., 2020). HSPA8 and HSPA2 are members of the heat shock protein 70 family (Gu et al., 2020) that act as chaperones to control protein folding when cells are exposed to stresses (Ouali et al., 2013). PDIA6 and ERP29 belong to the family of protein disulfide isomerases that serve as molecular chaperons for protein folding and their maturing into proper tertiary or quaternary structures (Rahman et al., 2022). In our study, the supplemented β-alanine and L-histidine may have stimulated oxidative stress (Moura et al., 2018), consequently deteriorating homeostasis within the cell (Rybka et al., 2011). To recover homeostasis, the pathways and proteins accordingly function, and ERP29 and PDIA6 proteins may promote the expression of HSPA8 and HSPA2 to combine free β-alanine with L-histidine, causing carnosine to increase in skeletal muscle.

Additionally, to the best of our knowledge, we identified the global biological processes and global molecular functions and cellular components of carnosine in the slow-growing chicken for the first time. All processes are related to maintaining homeostasis in muscles during their functions, including the cardiac muscle. In other words, the results of the cellular components reveal that carnosine functions in the sarcomere, thereby downregulating Titin and Connectin, and upregulating MYOM2, resulting in the production of passive force (Herzog et al., 2014). Furthermore, this preserves the M-band in the middle of the sarcomere (Lange et al., 2020) within the contractile, leading to the contraction of the muscle. Generally, muscles can function

when cells are in homeostasis (Roshni, 2021); however, anaerobic glycolysis during high-intensity muscle contraction leads to a decline in intracellular pH (Bowker et al., 2000) and denaturation, misfolding, and malfunction of proteins (O'Brien et al., 2012). Carnosine acts as a buffer when anaerobic glycolysis occurs in muscles (Hoffman et al., 2018) and inhibits the free radical activity by providing an electron to a radical molecule, leading to intracellular pH homeostasis (Boldyrev et al., 2013). Therefore, we can conclude that the maintenance of homeostasis and stress in the skeletal and cardiac muscle cells are the functions of carnosine. Interestingly, carnosine function significantly impacts cardiac muscle contraction, which is crucial for blood circulation to supply nutrients and oxygen into the cell and directly impacts chicken health.

Additionally, the results obtained from PCA showed positive loading between carnosine content and $pH_{45\ min}$ and described the secondary structure of proteins (β-turn and α-helix), proving that maintaining cellular homeostasis is a major function of carnosine. This association explains carnosine acting as a buffer to maintain intracellular pH homeostasis in early postmortem muscle (Boldyrev et al., 2013). Hence, pH_{45min} was not considerably low, and under the intracellular pH homeostasis, a stability of protein structure represented by high secondary structure of proteins (β-turn and α-helix) were found (Lu et al., 2011). A stable protein structure causes an improvement in water binding ability (Leygonie et al.,2012). These are desirable phenotypes pertaining to meat characteristics.

Thus, the explanations obtained from previous studies that heat shock proteins are in the same module and work together as they are related and PDIA6 and ERP29 are also in the same module since they belong to the family of protein disulfide isomerases (Rahman et al., 2022) have been proven. The other PPIs reveal an interaction among MYOM2, TTN, MYL10, Connectin, MYH7B, and FBP2, and they help in the maintenance of homeostasis and stress in the skeletal and cardiac muscle cells.

4.6 Conclusion

This study reveals that the pathways of protein processed in the ER and that of the proteins HSPA8, HSPA2, PDIA6, and ERP29 are involved in carnosine synthesis in the M. pectoralis major of the slow-growing KRC. Moreover, the global molecular function of carnosine, was also revealed, and carnosine possibly stimulates TTN,

Connectin, and MYOM2 expression to maintain homeostasis and stress in the skeletal and cardiac muscle cells. This finding indicates that carnosine plays a role in cardiac function that undoubtedly affects chicken health. Furthermore, the synthesis or consequence of carnosine function did not have any adverse effects on health and meat characteristics. These findings provide the confidence to take a step toward genetic improvement to enhance the ability of carnosine synthesis without adversely impacting the molecular function in chickens.

4.7 Acknowledgments

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CHAPTER VI

SUMMARY

The study can be concluded that both β-alanine and L-histidine are a limiting amino acid for carnosine synthesis, however, they do not any effect on anserine synthesis in slow-growing KRC breast meat. Moreover, the adding substrates of carnosine synthesis not adversely impact on growth performance of chickens, which it may be since the ability of carnosine to resist oxidative stress. On the other hand, supplementation with only β-alanine or L-histidine may negative effect on meat quality due to increase of β-sheet, β-turn, and aliphatic bending groups. Finally, higher carnosine contents do not adversely affect meat quality, improve meat texture, and alter the secondary structures of proteins.

For the results of proteomic profile concluded that the pathways of protein processed in the ER and the proteins HSPA8, HSPA2, PDIA6, and ERP29 are involved in carnosine synthesis in the *M. pectoralis major* of the slow-growing KRC, which this synthesis may occur when cells need to recover homeostasis. Moreover, the global molecular function revealed that increase of carnosine possibly stimulates TTN, Connectin, and MYOM2 expression to maintain homeostasis in muscles during their functions, including the cardiac muscle which affects the health of chickens.

Taken together higher carnosine synthesis may occur when cells need to recover homeostasis, leading to improved muscle function with no adverse effects on health or meat characteristics. Thus, these findings provide the confidence to step forward for genetic improvement to enhance the ability of carnosine synthesis without adverse impact on the molecular function in chickens. Moreover, this study can be suggested that the potential gene marker of HSPA8 and HSPA2 need to be investigated to improve carnosine levels in KRC meat and the factor of age on protein expression involved with carnosine synthesis in KRC needs to clarify.

APPENDIX

Supplementary table 1 Analytical value of amino acids content (%) in experimental diets in different growing phases.

Amino acid	Grower (22 to 42 days)[1]				Finisher (43 to 70 days)[1]			
	A	B	C	D	A	B	C	D
Asp	2.15	1.93	1.96	1.97	1.66	1.76	1.9	1.81
Thr	0.76	0.67	0.67	0.68	0.58	0.60	0.68	0.64
Ser	1.07	0.93	0.90	0.94	0.84	0.85	0.97	0.90
Glu	4.05	3.78	3.82	3.69	3.42	3.49	3.75	3.60
Pro	1.16	1.11	1.03	1.10	1.03	1.03	1.22	1.12
Gly	0.91	0.83	0.82	0.85	0.73	0.76	0.83	0.79
Ala	1.16	1.15	1.13	1.08	1.07	1.04	1.17	1.07
Cys	0.42	0.41	0.35	0.39	0.35	0.41	0.45	0.36
Val	0.97	0.95	0.94	0.94	0.81	0.84	0.93	0.90
Ile	0.84	0.82	0.83	0.82	0.69	0.72	0.79	0.76
Leu	1.76	1.67	1.74	1.72	1.53	1.52	1.71	1.60
Tyr	0.60	0.48	0.46	0.54	0.40	0.46	0.54	0.47
Phe	0.81	0.75	0.78	0.77	0.65	0.68	0.75	0.70
His	0.40	0.44	0.97	1.02	0.30	0.30	0.93	0.97
Lys	1.29	1.08	1.14	1.15	0.96	1.03	1.13	1.09
Arg	1.34	1.19	1.15	1.16	1.01	1.04	1.12	1.11

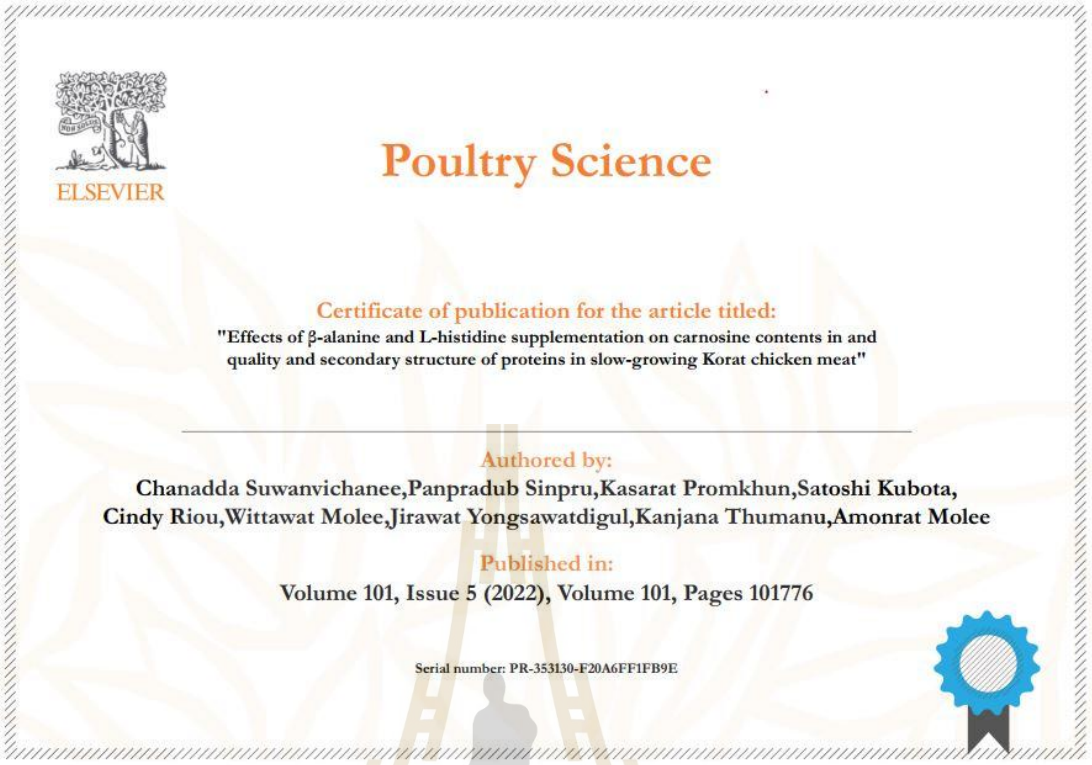
[1]Treatment groups are A (control), B (supplementation with 1.0% β-alanine), C (supplementation with 0.5% L-histidine), and D (supplementation with 1.0% β-alanine and 0.5% L-histidine), respectively.

Table S2 Proteins identified by LC-MS / MS analysis in breast meat samples from Low- and high-carnosine content of Korat chicken.

QR code for Supplementary file

Table S3 Summary information of mass spectrometry analysis.

QR code for Supplementary file



ELSEVIER

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BIOGRAPHY

Chanadda Suwanvichanee was born in September 27, 1989, at Saraburi, Thailand.In 2012, she graduated a Bachelor degree of Animal Science from Silpakorn University, Phetchaburi, Thailand and then received Master's degree of Animal Science from Maejo University, Chiang Mai, Thailand in 2016. In 2016, she decided to study her Ph.D. program in School of Animal Technology and Innovation under support of Suranaree University of Technology and Thailand Research Fund.

She presented oral and poster presentation including: 1) The 2nd International Conference on Native Chicken, Nakhon Ratchasima, Thailand [2019]; Oral presentation on the topic of Effect of dietary β alanine and L-histidine supplementation on growth performance and meat quality of female Korat chicken, 2) The world's poultry science association (Thailand branch), Bangkok, Thailand [2018]; Oral presentation on the topic of Effect of dietary β alanine and L-histidine supplementation on growth performance and meat quality of female Korat chicken, 3) The 9th National Animal Science Conference of Thailand, Nakhon Ratchasima, Thailand [2021] Symposium (Online) from Synchrotron Light Research Institute (Public Organization): Oral presentation on the topic of The study of biochemical changes of different carnosine content in Korat chicken meat by using Synchrotron-FTIR, 4) The 7th SUT International Colloquium on Agricultural Technology, Nakhon Ratchasima, Thailand [2022] Oral presentation on the topic of Proteomic profile involved with different carnosine content in slow-growing Korat chicken breast meat, 5) Mini conference PHC Siam BRC-Chic program, SBRI INSERM, Lyon, France [2022] Oral presentation on the topic of Proteomic profile involved with different carnosine content in slow-growing Korat chicken breast meat, and 6) Synchrotron-FTIR Reveals Biochemical Change and Meat Quality in Different Carnosine Content in Breast Meat of Slow-Growing Korat Chicken. Poster presented at The 10th SLRI Annual User Meeting 2022, Bangkok, Thailand.