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Evaluating the Efficacy of Whole Cooked Enriched Egg in Modulating Health-Beneficial Biological Activities

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EVALUATING THE EFFICACY OF WHOLE COOKED ENRICHED EGG IN MODULATING HEALTH-BENEFICIAL BIOLOGICAL ACTIVITIES

by

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The whole egg is known as nutritious food in the human diet with a wide reach due to its accessibility. It's highly bioavailable proteins and dietary compounds, and their health-beneficial properties make the egg a potential functional food. Still, little is known about how the production and processing methods can modulate its potential health-beneficial properties. To attend the knowledge gaps, the study established objectives look to evaluate the effect of the hen breed, egg enrichment, and cooking methods. The hen breed and egg enrichment maintained overall the egg quality. Confirming the capacity of the egg industry of improving the egg nutritional quality.

The cooking of different whole egg samples did not influence its digestibility but alter the peptide concentration in its hydrolysate. Similarly, the hen breed, enrichment, and cooking methods modulated the whole egg bioactivity. The antioxidant activity indicated standard White Leghorn (WLH) eggs were suitable for frying while Rhode Island Red (RIR) eggs had a higher activity when boiled irrespective of diet. RIR boiled showed the highest antihypertensive capacity, suggesting a synergistic effect. The enriched whole egg tended to a higher antihypertensive capacity. The peptide profile obtained through mass spectrometry analysis suggested the activities related to the peptide size, intensity, and structure. The study highlights empower the egg industry on adding value to the egg while educating consumers on how to obtain health-benefits from the whole egg.

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CHAPTER 1. INTRODUCTION

Hypertension accounts for one of the leading causes of morbidity and mortality worldwide, with a prevalence of 17.6% and 12.9% in the America continent and the United States, respectively (WHO, 2017b, 2017a). Hypertension is a multifactorial disease in which factors such as genetics and environment play a role in its mechanistic pathways. Such pathways include the Renin Angiotensin Aldosterone System (RAAS), increase oxidative stress and endothelial dysfunction, alteration in sympathetic nervous system, and vascular remodeling (Chapman et al., 2007; Esler, 2000; Montezano et al., 2015; Moore & Williams, 2002; Schrier, Masoumi, & Elhassan, 2010). Even though these mechanisms have characteristic pathways, evidence of system crosstalk indicates the upregulation of one system can modulate others (Barton, Shaw, D'uscio, Moreau, & Lüscher, 1997; Gonzalez-Vicente et al., 2016).

Several efforts have aimed to reduce the risk of cardiovascular diseases (CVDs), including hypertension, while lessening the burden on national health systems. Pharmaceuticals represent a partial solution that downregulates key actors of pathophysiological developmental pathways of hypertension. However, some of the pharmaceutical interventions have shown side effects such as dry cough, angioedema, and teratogenicity (Cooper et al., 2006; Gunkel, Thurner, Kanonier, Sprinzl, & Thumfart, 1996; Yesil, Yesil, Bayata, & Postaci, 1994). Food-derived dietary components could serve as alternatives with little or no side effects. Food-derived dietary components have shown biological activities that can modulate hypertension mechanisms. This alternative includes the role of functional foods exhibiting health-beneficial biological effects above

and beyond their known nutritional value (Lenoir-Wijnkoop, Jones, Uauy, Segal, & Milner, 2013; W. Yang, Gage, Jackson, & Raats, 2018).

Despite controversial cholesterol perception, the egg has been proposed as a potential functional food due to its excellent nutritional and biological properties, ameliorating the symptoms of hypertension or high blood pressure. The edible whole egg consists of egg white and egg yolk; components of both have shown antihypertensive and antioxidant activities. Peptides derived from egg proteins such as ovalbumin, ovotransferrin, lysozyme, and yolk proteins had shown ACE-inhibitory effects (Ewelina Eckert et al., 2019; Grootaert, Jacobs, et al., 2017; Majumder et al., 2015; Rao et al., 2012). Similarly, peptides from ovalbumin, ovotransferrin, lysozyme, and phosvitin had shown antioxidant activity (Dávalos, Miguel, Bartolomé, & López-Fandiño, 2004; X. Duan et al., 2014; Huang, Majumder, & Wu, 2010; H. Liu et al., 2006). Under certain conditions, the egg can exert beneficial biological properties, which makes it a potential functional food.

However, a more fundamental comprehension of how whole egg production and consumption modulate its biological effects is needed to determine its worth as a functional food, which can improve hypertension and associated CVDs. Egg production through laying hens is a well-established system with substantial research on egg production efficiency and egg quality. Nonetheless, there is a knowledge gap on how genetic variety and egg enrichment, through hen's feed, can modulate the health beneficial biological activity of different bioactive compounds. Most of the research in this area focuses on egg quality. Also, the processing effect, such as cooking, in modulating the egg's bioactive compounds has been addressed until recently (Majumder

& Wu, 2009; Nimalaratne, Savard, Gauthier, Schieber, & Wu, 2015; Wang, Liao, Nimalaratne, Chakrabarti, & Wu, 2018). Finally, there is less information about egg compounds' synergistic effect, if any, on human health.

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CHAPTER 2. LITERATURE REVIEW

INFLUENCE OF EGG BIOACTIVE COMPOUNDS ON HYPERTENSION-ASSOCIATED VASCULAR DISEASE: A REVIEW

Abstract

Hypertension is a multifactorial disease in which risk factors involve the Renin-Angiotensin-Aldosterone System, oxidative stress, endothelial dysfunction, and endothelin-1 as essential mediators. Bioactive compounds in functional foods have arisen as a natural alternative to reduce hypertension risk factors. However, the biological activity of the bioactive compounds in foods is often determined individually, overlooking the effect of the food matrix, processing, and gastrointestinal digestion. Egg protein-derived peptides, lipids, and carotenoids have shown antioxidant, antihypertensive, and anti-inflammatory activity, making the egg a nutritious and promising functional food. Carotenoids, omega-3 fatty acids, and peptides are the main contributors to these biological activities. Research has addressed the biological activities of the egg as a whole food until recently, elucidating how the whole egg production, cooking, and gastrointestinal digestion can increase its antioxidant and antihypertensive activity. This review collects studies up to date on the potential of functional foods such as whole egg as alternatives to reduce risk factors associated with the development of hypertension.

2.1. Introduction

Hypertension, a known global cardiovascular disease (CVD), is a chronic condition expressed as a combination of several risk factors such as 1) abnormal renin secretion and hyperactivation of the Renin-Angiotensin-Aldosterone system (RAAS), 2)

imbalance of nitric oxide (NO) generation in the endothelial cells due to increased levels of oxidative stress, and 3) increased activity of endothelin converting enzyme (ECE) and endothelin-1 in the endothelial cells (Chapman et al., 2007; Gryglewski, Palmer, & Moncada, 1986; Majumder & Wu, 2014; McMahon, Palomo, Brown, Bertenshaw, & Carter, 1993; Savoia et al., 2011; Susic, Varagic, & Frohlich, 2001). Along with these, several other risk factors influence the development of hypertension. However, in this review, the three will be addressed as critical mediators to reduce the risk of suffering hypertension.

Risk factors of CVDs, such as hypertension, are important in treating these conditions. Natural alternatives such as functional foods can ameliorate such risk factors. Additionally, research started to consider a different approach in which functional foods are a path to lower CVDs risk factors, possibly reducing CVDs burden on national health systems (W. Yang et al., 2018). Functional food capabilities on modulating risk factors rely on their biologically active compounds, produced or incorporated through several methods such as enrichment or processing. Compounds such as peptides, carotenoids, omega three fatty acids, phenols, vitamins, and minerals can provide functional foods with antioxidant, anti-inflammatory, antihypertensive, and immunomodulatory capacities.

Therefore, the potential use of hen egg as a functional food is promising. Its highly bioavailable protein and its health-beneficial properties make the egg one of the best protein sources in the food industry (Lee et al., 2009; Meram & Wu, 2017; Seuss-Baum, 2007). The egg low-cost makes it an ideal food to meet people's nutritional needs. It can meet high values of the recommended dietary allowance (RDA) for vitamin K, vitamin B_{12}, selenium, and reach 10% of the RDI for protein, vitamins A, D, E, B_2,

pantothenic acid, iron and zinc (Seuss-Baum, 2007). Therefore, each of the egg components plays a role in meeting these nutritional requirements. Due to its cholesterol content and triglycerides, the egg has been held responsible for contributing to cardiovascular diseases. Nonetheless, the literature review indicates that under moderate carbohydrate restriction and a three-egg/day consumption during 12 weeks did not influence plasma LDL-C levels in comparison to a cholesterol-free egg substitute in overweight individuals (Clayton, Fusco, & Kern, 2017; Mutungi et al., 2008).

2.2. Hypertension pathways and mechanisms

2.2.1. Renin-Angiotensin-Aldosterone System (RAAS) in vascular diseases

One of the hypertension's main signaling pathways is the Renin-Angiotensin-Aldosterone System (RAAS), responsible for regulating blood pressure in the human body (Oparil & Haber, 1974). The system, regarded as a hormonal cascade, performs a central role in modulating vascular tone, sodium secretion, and retaining fluid volume (Kaschina, Steckelings, & Unger, 2014). The kidney juxtaglomerular (JG) cells secrete renin in an inactive form, which is cleaved by microsomes to produce prorenin (Nehme, Zouein, Zayeri, & Zibara, 2019). Prorenin is a 406 amino-acid long protein which is released into the bloodstream or cleaved by cathepsin B and non-proteolytically by renin/prorenin receptors to produce renin in its active form, later stored in JG cells granules (Nehme et al., 2019; Patel, Rauf, Khan, & Abu-Izneid, 2017). Active renin, a 304 amino acid long protein, is released into the bloodstream in response to low arterial blood pressure, sodium imbalance, and sympathetic nervous system activity (Patel et al., 2017). While in the bloodstream, renin cleaves angiotensinogen to produce Angiotensin-I (Ang-I) (Muñoz-Durango et al., 2016).

Angiotensinogen is a 118 amino acid long α-globulin protein produce mainly in the liver. Additionally, angiotensinogen is expressed in the heart, blood vessels, kidneys, and adipose tissue. Its release is controlled by inflammation, insulin, estrogen, glucocorticoids, thyroid hormone, and Angiotensin-II (Ang-II) (Patel et al., 2017; Verdecchia, Angeli, Mazzotta, Gentile, & Reboldi, 2008). Angiotensinogen is cleaved when a 7-amino acid residue inserts in the active site of renin, causing the hydrolysis of the Leu10-Val11 bond, generating the decapeptide Ang-I (Verdecchia et al., 2008). Ang-I is then further cleaved in the lungs capillaries, endothelial cells, and kidney epithelial cells (Patel et al., 2017). These tissues contain angiotensin-converting enzyme 1 (ACE-1), a membrane-bound and zinc-dependent carboxypeptidase (Nehme et al., 2019; Patel et al., 2017; Verdecchia et al., 2008). ACE1 cleaves Ang-I between Phe8-His9, removing two C terminal amino acids (Leu10-His9) to form Ang-II (Patel et al., 2017; Tan, Liao, Zhou, Mei, & Wong, 2018). Consequently, Ang-II binds with the G-protein-coupled receptor angiotensin II type 1 receptor (AT_1R) and angiotensin II type 2 receptor (AT_2R). AT_1R triggers vasoconstriction, sodium retention, weak bronchoconstriction, inflammation via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), nicotinamide adenine dinucleotide (NADH)/nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and reactive oxygen species (ROS) production. It also induces apoptosis via toll-like receptor 4, and lung fibroblast proliferation (Kaparianos & Argyropoulou, 2011; Nehme et al., 2019; Tan et al., 2018).

Consequently, the system can diverge into alternatives pathways, including the angiotensin-converting enzyme 2 (ACE-2) and the aldosterone hormone pathway. ACE2, a membrane-anchored carboxypeptidase, cleaves Ang-I into the nonapeptide Ang (1-9)

by the C-terminal Leu10 (Donoghue et al., 2000; Santos et al., 2018). Reports indicated that Ang (1-9) modulates the cardiovascular system, possibly through AT_2R. In cardiomyocytes, Ang (1-9) exhibited anti-hypertrophic effects, which were blocked by PD123319 (AT_2R antagonist) (Mckinney, Fattah, Loughrey, Milligan, & Nicklin, 2014). Recently, a study has shown that Ang (1-9) reduced hypertension and increase vasodilation in DOCA-salt hypertensive rats, partially inhibited by PD123319. However, the study indicated this effect was independent of AT_2R and mitochondrial assembly (*mas*), suggesting it was due to the anti-inflammatory effect in the heart, aortic wall, and kidney (Gonzalez et al., 2018). Additionally, ACE2 cleaves Ang-II Pro7 and Phe8 bond into the heptapeptide Ang (1-7) (Santos et al., 2018; Tan et al., 2018; Tipnis et al., 2000). Another pathway of Ang (1-7) production is through the cleavage of Ang I by human neutral endopeptidase 24.11 (Jackman et al., 2002; Welches, Bridget Brosnihan, & Ferrario, 1993). The Ang (1-7) peptide attaches to the *mas* receptor inhibiting the growth of cardiac myocytes while inducing vasodilator, antiproliferative, anti-inflammatory, and antifibrotic effects (Simões E Silva, Silveira, Ferreira, & Teixeira, 2013; Tallant, Ferrario, & Gallagher, 2005). Even though studies suggest, under certain conditions, Ang (1-7) interaction with AT_2R have similar effects to *mas* receptor such as vasodilation, such effects are questionable due to unspecific binding to AT_2R or hetero-/homodimerization of the *mas* with AT_2R (Nehme et al., 2019; Ren, Garvin, & Carretero, 2002; Shimada et al., 2015; Villela et al., 2015). Besides, heterodimerization between *mas* and AT_1R blocks the effect of Ang-II, suggesting alternative pathways for vasodilation (Santos et al., 2018; Villela et al., 2015).

Aldosterone, a steroid hormone secreted by the adrenal gland cortex, role in hypertension comprises its ability to maintain the sodium-potassium homeostasis. The homeostasis is achieved by sodium reabsorption through proximal kidney tubules, losing potassium in the process (Cannavo et al., 2018; Patel et al., 2017; Spät & Hunyady, 2004). Aldosterone secretion initiates as Ang-II promotes the gene CYP11B2, responsible for the enzyme aldosterone synthase production in the zona glomerulosa of the adrenal cortex (Fuller & Young, 2016). Compounds such as potassium and adrenocorticotrophic hormone (ACTH) play a role in aldosterone synthase expression (Hargovan & Ferro, 2014). Afterward, aldosterone interacts with the mineralocorticoid receptors (MR), which is a transcription factor recognize by hormone response elements (Hargovan & Ferro, 2014). MR translocates to the nucleus and upregulates the transcription of the epithelial sodium channel (ENaC) genes and serum-glucocorticoid kinase (sgk) (Muñoz-Durango et al., 2016). Moreover, aldosterone promotes Na^+/K^+-ATPase activity and channel-inducing factor (CHIF) (Fuller & Young, 2016). The latter one is responsible for increasing the affinity of Na^+/K^+-ATPase for sodium. Finally, potassium balance is achieved through the α subunit of K^+-ATPase, regulated by dietary potassium and corticosteroids (Fuller & Young, 2016). As a result, regulation of blood pressure is due to the electrolyte homeostasis. Finally, MR activation modulates NOX, responsible for ROS generation (Hargovan & Ferro, 2014). Therefore, reducing nitric oxide and aiding the development of endothelial dysfunction discussed next.

2.2.2. Oxidative stress in vascular diseases

One characteristic of vascular diseases, such as hypertension, is endothelial dysfunction. A characteristic of this condition is an imbalance endothelium homeostasis

between vasodilation and vasoconstriction (Davignon & Ganz, 2004). Consequently, vascular smooth muscle cells (VSMC) and endothelial cells undergo vasoconstrictor, mitogenic, profibrotic, promigratory, and proinflammatory phenotype modulated by ROS (Montezano et al., 2015). Several enzymatic sources generated endothelial ROS. Nonphagocytic NOX catalyzes O_2 reduction, thus generating superoxide anion (O_2^-) (J.-M. Li & Shah, 2004; Montezano et al., 2015). Additionally, NOX is modulated by hypertension factors such as Ang-II, aldosterone, and endothelin-1 (ET-1), contributing to the homeostasis imbalance (Montezano et al., 2015). Other factors contributing to ROS generation include xanthine oxidase, uncoupled endothelial NO synthase (eNOS), mitochondria, Cytochrome P-450, and ROS source interaction (J.-M. Li & Shah, 2004; Montezano et al., 2015). Furthermore, a RAS crosstalk enables Ang-II to react with NOX via protein kinase C (PKC), producing superoxide anion (O_2^-), which inactivates nitric oxide (NO) and decrease its bioavailability(Majumder & Wu, 2014; Mehta & Griendling, 2007; F. Zhang, Ren, Zhao, Zhou, & Han, 2016).

NO is involved in vasodilation through several proposed pathways in the endothelial cells and VSMC. Endothelial cells produce NO through the eNOS conversion of L-arginine to NO and L-citrulline (Behrendt & Ganz, 2002; Davignon & Ganz, 2004). eNOS is activated after intercellular calcium influx from vasodilator agonists liberates the inhibitor caveolin-1 from calmodulin in the cell membrane invaginations known as caveolæ. Additionally, Tetrahydrobiopterin (BH_4) and NADPH function as eNOS cofactors for NO production (Davignon & Ganz, 2004). Neuronal NOS (nNOS) and inducible NOS (iNOS) are NOS isoforms. nNOS is express in endothelial cells, while iNOS cell expression is modulated by inflammatory cytokines and bacterial

lipopolysaccharide (LPS) (Forstermann et al., 1991; Majumder & Wu, 2014). Finally, NO diffuses through endothelial cell membranes into VSMC, activating soluble guanylyl cyclase, which starts off guanosine triphosphate conversion into cyclic guanosine monophosphate responsible for vasodilation (Behrendt & Ganz, 2002; Stankevicius, Kevelaitis, Vainorius, & Simonsen, 2003). NO also diffuse to red blood cells, where oxyhemoglobin converts it to nitrate. Such reactions limit NO half-life to less than a second (Butler, Megson, & Wright, 1998; Joshi et al., 2002).

However, ROS interaction with NO produces peroxynitrite ($ONOO^-$), a strong oxidant with a slow reaction rate capable of diffusing to a lesser extent compared to NO (Pacher, Beckman, & Liaudet, 2007). Peroxynitrite's biological targets include proteins, lipids, and nucleic acids (Pacher et al., 2007). Such compounds damage, present in cell membranes, cause inflammation and precedes cell death (Majumder & Wu, 2014). Therefore, peroxynitrite formation reduces NO bioavailability, participating in endothelial dysfunction while triggering vascular remodeling and inflammatory pathways (Pacher et al., 2007). Several approaches, such as antioxidant-rich diets, ACE inhibitors, and eNOS cofactor supplementation, have shown positive effects in diminishing hypertension (Pacher et al., 2007). Additionally, observational studies in populations showed an inverse correlation between plasma antioxidant levels and blood pressure levels.

2.2.3. Endothelin-1 in vascular diseases

The endothelin system's role in hypertension is derived from its key component, ET-1, regarded as one of the most potent vasoconstrictors up to date (Davenport et al., 2016; Granger, Spradley, & Bakrania, 2018). Physiological and pathophysiological

conditions such as shear stress, hypoxia, thrombin, and Ang-II upregulates ET-1 predecessor, pre-proendothelin-1 (preproET-1) (Davenport et al., 2016). PreproET-1 is a 212 amino acid protein cleaved by a signal peptidase removing the 17 amino acid signal, conforming proET-1. Furin-like endopeptidase cleaves the C and N terminal removing 35 and 122 amino acids, respectively, remaining the 41 amino acid peptide big endothelin-1 (big ET-1) (Davenport et al., 2016; Kedzierski & Yanagisawa, 2001). Finally, big endothelin is cleaved by endothelin converting enzyme 1 (ECE-1) at the Trp21-Val22 producing the 21 amino acid peptide defined as ET-1 (Ichikawa et al., 2010; Inoue et al., 1989; Miner-Williams, Stevens, & Moughan, 2014). ECE-1 has been isolated from the endothelium and found in VSMC, macrophages, and neurons (Houde, Desbiens, & D'Orléans-Juste, 2016). ET isoforms have been found, such as ET-2 and ET-3 derived from ECE-2 and ECE-3 activity (Inoue et al., 1989). However, *in vitro* studies showed that ECE-1 and ECE-2 affinity for big ET-1 is higher over big ET-2 and big ET-3, potentially making ET-1 the main actor in the endothelin system (Kedzierski & Yanagisawa, 2001).

ET-1 consequently binds to two G-protein-coupled receptors, ET-1 Type A (ET_A) and Type B (ET_B) (Granger et al., 2018). ET_A, located in VSMC, induce constriction through a Ca^{2+} cell influx and ROS generation (Dabbs Loomis, Sullivan, Osmond, Pollock, & Pollock, 2005; Granger et al., 2018; Kowalczyk, Kleniewska, Kolodziejczyk, Skibska, & Goraca, 2015; Schroeder et al., 2000). Whereas, ET_B receptor contributes to vasodilation by upregulating NOS, responsible for NO production (Granger et al., 2018; Shihoya et al., 2016; Taylor, Gariepy, Pollock, & Pollock, 2003). Also, ET_B receptor has shown, in transfected Chinese hamster ovary cells, to bind ET-1 irreversibly and follow

the internalization pathway into lysosomes for degradation. The degradation pathway is due to the serine cluster presence in ET_B, responsible for the stable binding of arrestin and therefore targeted by lysosomes (Bremnes et al., 2000; Shihoya et al., 2016).

Among external factors that modulate the endothelin system include endotoxins and Ang-II (Gopalakrishna, Pennington, Karaa, & Clemens, 2016; Hoesel & Schmid, 2013). Previous reports indicated vascular endothelial cells subjected to LPS endotoxin decreased tetrahydrobiopterin (BH_4), responsible for eNOS homodimer stability. Therefore, eNOS undergo monomerization by ET-1. The eNOS oxygenase domain reduces its active site heme-oxy complex. As a consequence, eNOS generated superoxide radical contributes to a lower NO availability in smooth muscle while activating constriction (Gopalakrishna et al., 2016). Additionally, *in vivo* studies in WKY rats showed an increase of ET-1 in VSMC as well as ECE activity in aorta due to Ang-II infusion (Barton et al., 1997; Kohan & Barton, 2014). These pathways show a dynamic system with possible crosstalks responsible for vasoconstriction and blood pressure regulation.

2.3. Effect of food-derived compounds on hypertension

Efforts had been made to research natural alternatives in order to avoid secondary side effects of current pharmaceuticals. A potential alternative has been seen in bioactive compounds, phytochemicals, and peptide from food sources as they present antioxidant, ACE-Inhibitory, and ECE-inhibitory activity.

2.3.1. Food compound and peptides antioxidant activity

The antioxidant capacity of food has been researched on numerous fruits and vegetables, as well as dairy and meat. Food like quinoa, amaranth, banana, berries, and

potato to name some (Akyol, Riciputi, Capanoglu, Caboni, & Verardo, 2016; B. Singh, Singh, Kaur, & Singh, 2016; Skrovankova, Sumczynski, Mlcek, Jurikova, & Sochor, 2015; Tang & Tsao, 2017) had shown to contain antioxidant properties. Food peptides derived from parent proteins exhibit antioxidant activity. These can be derived from a wide variety of sources as meat, milk, eggs, fish, zein, almond, and soybean protein (Lorenzo et al., 2018). Proposed characteristics of these peptides are the presence of Q at the N-terminal, W at the C-terminal, presence of amino acids K, M, H, W and Y as metal ions chelators, and E and N amino acid as free radical inhibitors (R. E. Aluko, 2015; Peńa-Ramos & Xiong, 2001).

2.3.2. Food proteins antihypertensive activity

Hypertension is a multifactorial disease. However, natural food inhibitors downregulate the RAAS enzyme ACE. Despite food components, variety, inhibition of ACE has been seen from food hydrolysates and peptides. The hydrolysis and release of the peptides from its parent protein are achieved through autolysis, fermentation, acid hydrolysis, enzymatic hydrolysis, and gastrointestinal digestion (García, Puchalska, Esteve, & Marina, 2013; Orio et al., 2017). The efficiency of inhibiting ACE enzyme depends on the peptide characteristics. Dipeptides structure requires bulky and hydrophobic side chains amino acids, while tripeptides structure is composed of an aromatic amino acid in the carboxyl terminus, positively charged amino acids in the middle, and hydrophobic amino acids in the amino terminus (Jianping Wu, Aluko, & Nakai, 2006b). Furthermore, antihypertensive activity seen on long-chain peptides may be due to their hydrolysis by proteases in the gastrointestinal tract or plasma (Rotimi E Aluko, 2019).

2.3.3. Food Proteins ECE-1 inhibitory activity

The regulation of the endothelin cells function through ECE-1 inhibition has been seen from food proteins peptides and phytochemicals. Initial research found pepsin digest from Bonito Pyrolic appendix and beef had ECE inhibitory potential (Okitsu, Morita, Okada, Yokogoshi, & Kakitani, 1995). The ACE-inhibitor lactokinin peptide derived from β-lactoglobulin tryptic digestion, ALPMHIR, showed a reduction of ET-1 release in porcine aortic endothelial cells. The study did not assess ECE-1 inhibition directly, but it was considered as a possible mechanism to reduce ET-1 release (Maes et al., 2004). Recent studies showed peptides from milk lactoferrin, and dry-cured ham byproducts had ECE-1 inhibitory properties (Fernández-Musoles et al., 2010, 2013; Gallego, Mora, Hayes, Reig, & Toldrá, 2019). Such studies indicated the ability of peptides to modulate the activity ACE-1, ECE-1, and dipeptidyl peptidase-IV (Gallego et al., 2019).

2.3.4. Polyunsaturated fatty acids biological activity

The polyunsaturated fatty acids (PUFA) content of a food may vary depending on its source. Their carbon chain length and configuration are characteristic of its source. PUFA have omega-3 and omega-6 configuration. Research showed PUFA ability to regulate platelet function and thrombosis, decrease inflammatory cytokines, neuroprotective and partial neurorecovery, and improve artery endothelium-dependent dilation (Adili, Hawley, & Holinstat, 2018; Endo & Arita, 2016; Goodfellow, Bellamy, Ramsey, Jones, & Lewis, 2000; Kerdiles, Layé, & Calon, 2017; Nodari et al., 2011). Besides contributing to these biological functions, polyunsaturated fatty acids had also modulated the hypertension system in previous research. A study with 115 newly diagnosed hypertension patients with omega-3 consumption had a higher concentration of

plasma PUFA and showed a trend to a lower peripheral and central blood pressure (Bagge et al., 2017).

2.3.5. Lutein and zeaxanthin biological activity

Carotenoids comprise a varied group with more than 600 molecules (Jomova & Valko, 2013). Carotenoids are divided into two main groups based on their functional groups: xanthophylls and carotenes. Xanthophylls contain lutein and zeaxanthin, while carotenes include α-carotene, β-carotene, and lycopene (Jomova & Valko, 2013; Milani, Basirnejad, Shahbazi, & Bolhassani, 2017). Some of the main sources of xanthophylls include egg yolk, corn, orange pepper, kiwi, grapes, spinach, orange juice, zucchini, squash, kale, broccoli, among others (Abdel-Aal, Akhtar, Zaheer, & Ali, 2013; Perry, Rasmussen, & Johnson, 2009; Sommerburg, Keunen, Bird, & Van Kuijk, 1998). However, xanthophyll bioavailability is higher in products like an egg when compared to vegetable sources or supplements (Chung, Rasmussen, & Johnson, 2004). Research has shown xanthophyll molecules such as lutein and zeaxanthin increase macular pigment density, protect from photooxidative damage and inflammation, and reduce biological oxidative stress (Bian et al., 2012; Giordano & Quadro, 2018; R. Liu et al., 2015; Perrone et al., 2014). The carotenoid antioxidant mechanism is based on the quenching of singlet molecular oxygen and peroxyl radicals (Stahl & Sies, 2003; Young & Lowe, 2001). The singlet oxygen energy is relocated to the carotene molecule yielding a ground state oxygen and a triplet state carotene. Later, the carotenoid dissipates its energy into its solvent (Baltschun et al., 1997; Stahl & Sies, 2003). Additionally, the peroxyl radicals generated in an organism through lipid peroxidation is quenched by the carotenoids. In this process, the carotenoid's lipophilicity and radical scavenging property possibly

enable the formation of radical adducts from peroxyl radicals. As a result, the carotenoid structure change to a resonance stabilized carbon-centered radical (Burton, 1989; Stahl & Sies, 2003).

2.4. Egg components and their bioactivity

Foods can confer one or more biological activities, depending on their composition. Egg, as a complex food matrix, has previously reported several biological activities from its components and as whole food. Several egg components had shown antihypertensive and antioxidant effects individually and as a whole.

2.4.1. Egg white biological activities

Composed mainly of water with a protein content of 10%. Egg white comprehends some of the egg's most important functional and bioactive proteins. The main egg white proteins such as ovalbumin, ovotransferrin, ovomucoid, ovomucin, and lysozyme reported antimicrobial, antihypertensive, immunomodulatory, anti-inflammatory, and antioxidant activities (Andersen, 2015; Kovacs-Nolan, Phillips, & Mine, 2005). Ovalbumin glycosylation with galactomannan polysaccharide, ovotransferrin iron-binding property, and lysozyme binding property with advance glycation end-products proved to reduce free radicals (R171, r389, r390) (Ibrahim, Hoq, & Aoki, 2007; H. Liu et al., 2006; S. Nakamura & Kato, 2000). Also, egg white proteins subjected to a degradation or hydrolysis process can enhance its antioxidant properties and even release antioxidant peptides from its parent protein (Shen, Chahal, Majumder, You, & Wu, 2010).

Egg white peptides also confer ACE-inhibitory activity. Ovotransferrin peptides hydrolyzed with thermolysin and pepsin yielded the antihypertensive peptides IQW and

LKP (R7)(Majumder & Wu, 2011). Both peptides also prove to reduce blood pressure in spontaneously hypertensive rats (SHR)(Majumder et al., 2015). Hydrolysis through gastrointestinal enzymes such as pepsin, trypsin, chymotrypsin, or pancreatin also yields ACE-inhibitory peptides. When subjected to gastrointestinal hydrolysis, lysozyme release ACE-inhibitory peptides VAW and MKR with IC_{50} values of 2.86 ± 0.08 and 25.7 ± 0.2 μM, respectively (Rao et al., 2012). Finally, it is important to mention that the absorption and bioavailability of theses bioactive peptides are improved when consumed through a food matrix (Grootaert, Jacobs, et al., 2017).

2.4.2. Yolk biological activities

Lipids, proteins, vitamins, and minerals are all encountered in the egg yolk. As ovaries' follicles engulf an ovum, it contains these necessary elements for the formation and development of hen's embryo. Therefore, egg yolk naturally contains biologically active compounds. Egg yolk proteins including low-density lipoproteins, high-density lipoproteins, livetin, phosvitin, and biotin-binding proteins conveyed antioxidant, antihypertensive, anti-inflammatory, and antimicrobial activities (E. Eckert et al., 2013; Ewelina Eckert et al., 2014; Yoshii et al., 2001; Zambrowicz, Dabrowska, Bobak, & Szoltysik, 2014).

Research has been done on carotenoid and omega-3 fatty acids enrichment in egg yolk to increase its nutritional value and as a result, its biological activity. Egg yolk antioxidant capacity can be derived from compounds such as proteins, peptides, amino acids, and carotenoids. Egg yolk free aromatic amino acids have been identified as one of the main contributors to egg yolk antioxidant activity. However, amino acids are

susceptible to the Maillard reaction during different cooking methods (Nimalaratne, Lopes-Lutz, Schieber, & Wu, 2011).

Furthermore, eggs enriched in carotenoids and omega-3, has shown superior antioxidant activity when compared to White Leghorn eggs (Nimalaratne, Schieber, & Wu, 2016). The main carotenoids present in the egg are the xanthophylls lutein, zeaxanthin, and in lower amount canthaxanthin and β-apo-8′-carotenoic acid ethyl ester. Similar to free amino acids, carotenoids are susceptible to cooking methods as their content is decreased when compared to the uncooked egg (Nimalaratne, Lopes-Lutz, Schieber, & Wu, 2012).

Yolk oligopeptides obtained after enzymatic hydrolysis confers its antihypertensive capacity. The oligopeptides had a moderate action on regulating blood pressure on SHR with an IC_{50} value of 1.22 ± 0.08 mg/mL determined in vitro (Yoshii et al., 2001). Further research used unconventional enzyme from *Cucurbita ficifolia* for the hydrolysate production. The yolk hydrolysate IC_{50} value determined in vitro was 482.5 µg/mL with no cytotoxic activity on human cell lines(Ewelina Eckert et al., 2014). As indicated, the preparation and hydrolysis of the yolk proteins are determinant for its antihypertensive capacity.

2.4.3. Whole egg biological activities

The whole egg is known for its high nutritional value. Whole egg components such as egg white and egg yolk had shown potential biological activity *in vitro* and *in vivo*. However, the significance of these bioactivities must consider a holistic approach as the egg is mostly consumed as a complex food matrix compose of egg white and egg yolk. It is important to consider the processing effect of cooking, as it could enhance or diminish

the activity of bioactive compounds. Finally, the bioactive compounds bioactivities might differ as the complete matrix undergo gastrointestinal digestion. This final step could alter the bio-accessible elements absorbed in the gastrointestinal tract.

Initial research on this approach evaluated the whole egg hydrolysate antioxidant and antihypertensive capacity. Remanan and Wu (2014) showed that cooking treatments such as boiling and frying on egg white and whole egg showed no difference when compared to its fresh counterpart. However, the cooking treatment did reduce egg yolk antioxidant capacity. Additionally, the simulated gastrointestinal digestion was seen to enhance the egg antioxidant capacity derived from peptide and amino acid release (Remanan & Wu, 2014).

The whole egg antihypertensive capacity was evaluated. The whole egg was subjected to frying and boiling and posteriorly hydrolyzed through gastrointestinal digestion. It was shown that fried whole egg digest had the highest ACE inhibitory IC_{50} value (0.009 mg protein/mL). Different tripeptides were identified and indicated a possible in vivo effect after absorption (Majumder & Wu, 2009). Posteriorly, the fried whole egg digest effect was evaluated in vivo trough SHR. It was shown that a decrease in blood pressure was achieved at a dose of 1000 mg/kg (Majumder, Panahi, Kaufman, & Wu, 2013). Finally, the long-term effect was analyzed by determining its effect on blood pressure, plasma lipid profile, and oxidative stress in SHR. Like the last study, fried whole egg digest (FWED) was able to reduce blood pressure but not its unhydrolyzed counterpart. Blood pressure reduction was followed by NO-dependent vasorelaxation and plasma Ang II reduction. FWED also reduce the triglycerides levels in plasma while

reducing oxidative stress (Jahandideh et al., 2014). This set of studies have shown the potential of the whole egg as a functional food, and in ameliorating CVDs risk factors.

2.5. Concluding Remarks

Alternatives in addressing CVDs risk factors have turned the attention to functional foods as a potential solution due to the side effects of pharmaceutical interventions. The risk factors have unique mechanism pathways, but pathways crosstalk evidence suggests a connected and dynamic network in our organism. It is important to modulate the mechanism pathways key actors in order to ameliorate their effects, which could be achieved through bioactive compounds found in several foods. The biological activity of these compounds is inherent to the composition and chemistry of the food. While several foods have beneficial biological activity, an alternative presenting two or more beneficial effects is of great advantage, which could simultaneously modulate the multiple pathways.

As previously discussed in this review, the whole egg contains high protein content and several vitamins important for our RDA. However, the production system and processing can enhance the egg profile into a potential functional food. The whole egg has previously shown several bioactivities, inherent to its complex matrix and rich composition. Nonetheless, to better understand its potential as a functional food, it is important to evaluate which factors enhance its biological activities. The practical application of functional foods also must include the human gastrointestinal system. Once consumed, the bio-accessibility and bioavailability of the bioactive compounds must be determined for the functional food to work successfully. Therefore, ameliorating CVDs risk factors successfully.

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CHAPTER 3. PHYSICAL CHARACTERIZATION OF STANDARD AND ENRICHED EGGS AND EXPERIMENTAL DESIGN

Abstract

Egg production is an essential component of the United States poultry industry. External and internal factors determine egg quality. Modern trends include egg product enrichment, which should not comprise its quality. In this study, White Leghorn (WLH) and Rhode Island Red (RIR) hen breed were fed with a corn-soybean meal-based diet and an enriched diet of a corn-soybean meal-based containing flaxseed and marigold extract as a source of omega-3 fatty acids and lutein, respectively. It was observed that hen breeds influenced egg white, yolk, eggshell percentages, and Haugh units. Egg enrichment increased egg white percentage while decreasing eggshell strength compared to the control. Overall, egg enrichment maintained the egg quality within the hen breeds. Enriched eggs with enhanced nutritional quality could further benefit the consumer's nutrition.

3.1. Introduction

Egg production by laying hens is an important sector in the poultry industry in the United States and worldwide. In the United States, as of August 2019, table eggs accounted for 8.25 billion. While all layers in the United States in September 2019, totaled 393 million (NASS & USDA, 2019). Layer lines such as White Leghorn and Rhode Island Red are one of the main hen breeds for commercial and backyard egg production, respectively (Lukanov, Genchev, & Pavlov, 2015). Hen breeds are chosen based on traits such as high egg production, eggshell breaking strength, shell thickness, egg weight, shape, Haugh units, and yolk color associated with external and internal

quality (Singh, Cheng, & Silversides, 2009). Additionally, White Leghorn hen breed is better adapted to cage production due to its docile behavior with a high feed conversion ratio. Rhode Island Red hen breed is better adapted to alternatives production systems such as cage free or free range due to its territorial behavior.

The egg is known for its nutritional quality, containing all essential amino acids, vitamins, and minerals. Additionally, the nutritional value of the egg can be modified based on its feed components (Novak & Scheideler, 2001). Feed components such as flaxseed, fish oil, dried tomato waste or corn distiller dried grains provide the hen with valuable nutritional compounds such as omega-3 fatty acids and carotenoids (Panaite et al., 2019; Scheideler & Froning, 1996; Shin et al., 2016). The addition of these nutrients should maintain or in some cases, improve egg quality. Several egg characteristics are considered to evaluate its external and internal quality. External quality factors include egg size, shell color, shell breaking strength, shell percentage, and thickness, among others (Sharaf Eddin, Ibrahim, & Tahergorabi, 2019). Yolk quality, the integrity of the vitelline membrane, and albumen quality comprise internal quality factors (Sharaf Eddin et al., 2019).

Therefore, as a result of supplementation, an egg with specific characteristics is obtained, which should comply with the industry quality and food safety requirements. Egg enrichment research has focused on the effect of enrichment on egg quality and in some cases evaluating different hen genetics. But the effect of the hen breed is not completely understood in aspects such as egg enrichment efficiency. Addressing this knowledge gap would be of great benefit for the egg and food industries which constantly search to deliver high quality eggs to the consumers. This chapter includes the

experimental design description of the thesis. However, this chapter aims to evaluate the effect of hen genetics and diet on egg external and internal quality.

3.2. Experimental Design

The experimental design is a factorial design with three factors. The factors of the study are hen breed, egg enrichment, and cooking methods. The breeds used are White Leghorn and Rhode Island Red, evaluating the genetic background effect on egg enrichment and biological activity. Hens were fed with a corn-soybean meal-based diet and a corn-soybean meal-based diet supplemented with flaxseed and ORO GLO®[1] to enrich the egg. The diet formulations are described in Table 1 and 2. Flaxseed incorporation provides omega-3 fatty acids, and ORO GLO® provides lutein from the marigold source. The enrichment factor will assess if there is any difference between different breeds efficiency in incorporating the supplement compounds and if there is any effect on the egg biological activity. The first two factors also evaluate any effect on egg external and internal quality. Finally, the cooking methods used are pan-frying and boiling. The cooking method evaluates if there is any difference in the egg biological activity based on its processing. The complete design has a total of eight different treatments.

[1] ORO GLO® was provided by Kemin Corporation.

3.3. Physical Characterization of Standard and Enriched White Leghorn and Rhode Island Red Eggs

3.3.1. Materials and Methods

3.3.1.1. Sample collection

Production of fresh white-shell and brown-shell eggs took place at the Poultry Building F at the University of Nebraska-Lincoln. Breeds include White Leghorn and Rhode Island Red hens as a source of genetic variation. The hens were 37 weeks old at the beginning of the trial. Hens were fed for 16 weeks with a corn-soybean meal-based diet and a corn-soybean meal-based diet supplemented with flaxseed and ORO GLO® as a source of omega-3 and lutein, respectively. Storage of the collected eggs was done 4 °C until further analysis without exceeding 21 days.

3.3.1.2. Egg components

The standard and enriched White Leghorn and Rhode Island Red eggs were weighed using a precision balance. Next, an egg separator on top of a beaker previously tared separated the egg albumen from the egg yolk. The egg albumen weight was recorded. The egg yolk weight was recorded after removing the chalazae. The shell weight was calculated by weight difference between egg weight minus yolk and albumen weight.

3.3.1.3. Egg Haugh unit

The egg was weighed and broken on a flat surface. A tripod micrometer was used to measure the thick albumen height at room temperature. The egg Haugh unit was calculated according to Equation 1.

Equation 1. Haugh unit calculation formula (Cherian & Quezada, 2016; Haugh, 1937).

$$Haugh\ Unit = 100 \times \log(h - 1.7w^{0.37} + 7.6)\ [1]$$

Where h is the observed height of the egg white in millimeter and w the weight of the egg in grams.

3.3.1.4. Eggshell breaking strength

Eggshell breaking strength was analyzed with a TA.XT PLUS texture analyzer. An acrylic cylinder probe with 4 cm diameter and 20 mm tall was used to measure the break force of the eggshell in Newton. Eggs with fissure defects were removed from the analysis.

3.3.1.5. Statistical analysis

One-way ANOVA analyzed the effect of hen breed and diet on the egg components, egg Haugh units, and shell breaking strength through GraphPad Prism version 7.01 (La Jolla, California, USA). Significant differences were determined using the Tukey test at the level of $p<0.05$.

3.4. Results and Discussion

3.4.1. Egg Components

The egg components mean and the ANOVA *p*-values are described in Table 3.3. There was a significant difference between WLH control and WLH flax (58.83 vs. 59.62, respectively, $p<0.05$), as shown in Figure 1. Novak & Scheideler (2001) previously reported this increase in albumen percentage when hens were fed with a flaxseed supplemented diet. The increase in albumen in the hen breed Dekalb Delta was attributed to a decrease of yolk proportion (Novak & Scheideler, 2001). Also, the WLH control and

flax treatments showed a higher albumen percentage compared to their RIR counterpart. The hen genetic difference agrees with Knox and Godfrey (1933), who observed a difference for thick egg white. Even though the difference was identified in their study, it described that the thick egg white is a hereditary trait that can be modified by proper selection (Knox & Godfrey, 1934). This statement was later confirmed in a further study in which Rhode Island Red hens were bred to increase or decrease the proportion of thick egg white in 5 years (Knox & Godfrey, 1940).

There was no significant difference in yolk percentage between diets within the same line, as shown in Figure 2. However, the RIR eggs contained a relatively higher percentage of yolk than WLH eggs. Previous results reported that White Leghorn eggs had a higher yolk weight when compared to Rhode Island Red (Li et al., 1998). However, when considering the percentage of yolk to the egg weight, the yolk represented 34% with no differences between the hen breed. The yolk percentage obtained in this study may be a result of breeding practices selection to reduce the yolk content. Recent studies evaluating RIR egg and yolk weight showed, on average, a yolk percentage of 30 similar to our results (Mori, Takaya, Nishimura, & Goto, 2019). The supplementation of flaxseed in hens feed showed a reduction in yolk weight not observed in the current study. Fish oil and flaxseed at a 10% level had reported decreasing the yolk size, related to the regulation of hormonal metabolism by dietary fat in the laying hen (Scheideler & Froning, 1996).

Figure 3 shows that the shell percentage within the same hen breed was not different between the control and enriched diet. However, RIR eggs showed a higher shell percentage when compared to their WLH counterparts ($p<0.05$). Studies showed

that wet shell weight, as a percentage of total egg weight, was decreased by flaxseed supplementation. However, no difference was found after drying (Novak & Scheideler, 2001). This decrease was suggested to be due to the flaxseed laxative effect, increasing the rate of passage (Scheideler & Froning, 1996). Recent studies found no difference in eggshell percentage after feeding Hy-Line hens with a diet containing 10% flaxseed (Mattioli et al., 2017).

Eggs from WLH enriched diets had a higher weight compared to WLH control (55.58 vs. 58.93 g, respectively), as shown in Figure 4. Similarly, the egg from WLH enriched diets had a higher weight compared to RIR enriched (58.93 vs. 55.35 g, respectively). No significant difference was found between WLH control vs. RIR control nor RIR control vs. RIR enriched. Reports had shown a positive correlation between the total amount of albumen and the egg weight in both hen breeds, WLH and RIR (Knox & Godfrey, 1934). Such correlation would relate to the higher weight obtained in WLH enriched diet, which also showed a higher albumen percentage. The enriched diet did not affect the egg weight for the RIR line. These results agree with previous research, which supplemented Hisex line with up to 10% flaxseed without reporting a significant difference with the control (Yassein et al., 2015). The overall results of egg weight contrast with previous studies that observed a decrease in egg weight with a flaxseed diet, ascribed to of nutritional regulation hormone metabolism (Scheideler & Froning, 1996; Whitehead, Bowman, & Griffin, 1993). Finally, egg enrichment with lutein from marigold extract did not affect the egg weight (Leeson & Caston, 2004).

3.4.2. Egg Haugh unit

As shown in Figure 5, WLH eggs yield eggs with a higher Haugh unit when compared to their RIR counterparts. WLH control vs. RIR control (88.81 vs. 77.67, respectively) and WLH flax vs RIR flax (86.93 vs. 72.87, respectively) showed significant difference ($p<0.05$). WLH control and enriched samples showed no difference. However, RIR control had a higher Haugh unit when compared to RIR flax (77.67 vs. 72.87, respectively). Studies had analyzed the Haugh units from eggs laid by hen breeds crossed with RIR and WLH lines. The albumen index in Rhode Island Red crosses was lower as compared to White Leghorn crosses (Lukanov et al., 2015). Hen breeds such as Hy-Line and Lohman Brown fed with flaxseed at a 10% level did not affect the eggs Haugh units similar to our WLH results (Cherian & Quezada, 2016).

3.4.3. Eggshell breaking strength

Figure 6 shows the eggshell breaking strength of WLH control was the highest among the four treatments. WLH fed with flaxseed and lutein containing diet showed a lower eggshell breaking strength (48.33 vs. 43.82, respectively, $p<0.05$). In contrast, RIR control and enriched eggs, and their comparison against WLH enriched eggs did not show a statistical difference. The eggshell breaking strength had previously not been affected by diet supplementation of flaxseed and lutein (Panaite et al., 2019; Shin et al., 2016). Yassein et al. (2015) found that a decrease of eggshell thickness occurred due to flaxseed in the diet (Yassein et al., 2015). This parameter could contribute to the reduce eggshell breaking strength observed in the WLH line. These results are in accordance with previous studies indicating a lower shell percentage due to flaxseed, which can cause a laxative effect and decrease calcium intake (Scheideler & Froning, 1996).

Another factor to consider is the hen age, which has been shown to decrease eggshell thickness over 76 weeks of age (De Ketelaere et al., 2002). Finally, eggshell breaking strength is a trait with low heritability. Recent studies had a focus on genetics and molecular biology to better understand the factor influencing eggshell calcification in the chicken uterus. Eggshell matrix proteins played a role in eggshell strength. The proteins osteopontin and ovalbumin downregulation resulted in a low eggshell strength group of RIR (Zhang et al., 2015). Other factors important for eggshell thickness and its mechanical properties include the ion transporters important for egg mineralization (Duan et al., 2015).

3.5. Concluding Remarks

Hen diet supplementation with biologically active molecules is a validated strategy to increase the nutritional value of an egg. This enrichment should not compromise egg quality. In our study, the albumen percentage was increased in the WLH hen breed with the enriched diet. A hen breed effect was observed as WLH had a higher albumen percentage compared to their RIR counterparts. This trait had been previously observed as a hen breed effect. The yolk percentage was not decreased with the enriched diet in contrast to previous studies, and the RIR line showed a higher yolk percentage.

Shell weight was higher for RIR than WLH eggs with no effect from diet enrichment. An increase in egg weight is observed in WLH, previously related to a higher albumen percentage. Additionally, a higher Haugh unit was observed in WLH eggs compared to RIR. Eggshell breaking strength was higher in WLH control. Enriched eggs tended to have a reduce eggshell breaking strength, possibly due to the flaxseed laxative effect, which could reduce Ca intake. Overall, the egg enrichment maintained the egg

quality as the quality from WLH and RIR agrees with previous research. Enriched eggs with enhanced nutritional quality could further benefit the consumer's nutrition.

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Tables

Table 3.1. Corn-soybean meal-based diet formulation.

Ingredients	Percentage
Fine ground corn	56.52
Soybean Meal-47%	26.98
Oil	3.49
Dicalcium Phos	2.01
Shell & Bone Builder	5.01
Limestone	5.01
Salt, White	0.48
PL Methionine	0.28
Pinnacle Premix	0.22

Table 3. 2. Corn-soybean meal-based enriched diet formulation.

Ingredients	Percentage
Fine ground corn	50.82
Flaxseed	10.00
Soybean Meal-47%	24.26
Oil	3.14
Dicalcium Phos	1.81
Shell & Bone Builder	4.50
Limestone	4.50
Salt, White	0.43
Oro-glow	0.10
PL Methionine	0.25
Pinnacle Premix	0.20

Table 3.3. Hen breed and enrichment effect on egg components.

Egg white percentage	Mean±SEM 1 (%)	Mean±SEM 2 (%)	*p*-value
WLH Std (1) vs WLH Enrh (2)	58.83±0.20	59.62±0.20	0.0353
WLH Std vs RIR Std	58.83±0.20	56.99±0.23	<0.0001
WLH Std vs RIR Enrh	58.83±0.20	57.51±0.24	0.0002
WLH Enrh vs RIR Std	59.62±0.21	56.99±0.23	<0.0001
WLH Enrh vs RIR Enrh	59.62±0.21	57.51±0.24	<0.0001
RIR Std vs RIR Enrh	56.99±0.23	57.51±0.24	0.3828
Egg yolk percentage	Mean±SEM 1 (%)	Mean±SEM 2 (%)	*p*-value
WLH Std vs WLH Enrh	27.50±0.19	27.04±0.18	0.3395
WLH Std vs RIR Std	27.50±0.19	30.41±0.22	<0.0001
WLH Std vs RIR Enrh	27.50±0.19	29.79±0.24	<0.0001
WLH Enrh vs RIR Std	27.04±0.18	30.41±0.22	<0.0001
WLH Enrh vs RIR Enrh	27.04±0.18	30.41±0.22	<0.0001
RIR Std vs RIR Enrh	30.41±0.22	29.79±0.24	0.1846
Egg shell percentage	Mean±SEM 1 (%)	Mean±SEM 2 (%)	*p*-value
WLH Std (1) vs WLH Enrh	13.67±0.09	13.34±0.12	0.642
WLH Std vs RIR Std	13.67±0.09	14.43±0.30	0.0488
WLH Std vs RIR Enrh	13.67±0.09	14.37±0.30	0.0932
WLH Enrh vs RIR Std	13.34±0.12	14.43±0.30	0.0012
WLH Enrh vs RIR Enrh	13.34±0.12	14.37±0.30	0.0036
RIR Std vs RIR Enrh	14.43±0.30	14.37±0.30	0.998

Note: SEM: standard error of the mean, WLH: White Leghorn, RIR: Rhode Island Red, Std: standard, Enrh: enriched.

Figures

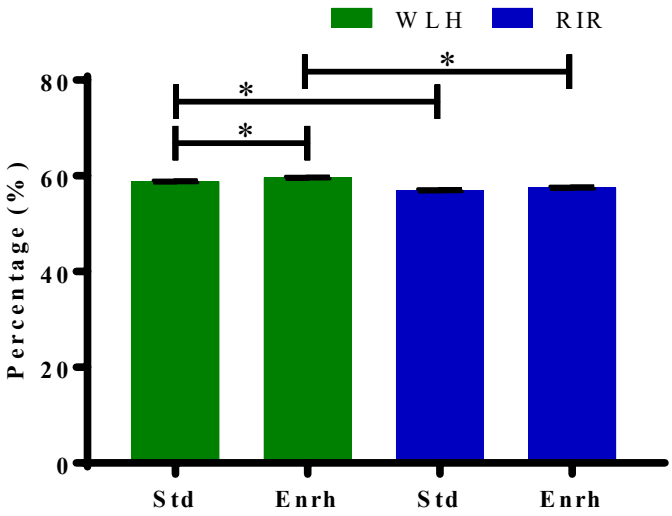

Figure 3.1. White Leghorn (WLH) and Rhode Island Red (RIR) albumen percentage from standard and enriched diets. Error bars represent standard error of the mean (SEM); *statistically significant difference (p<0.05, one-way ANOVA, Tukey test).

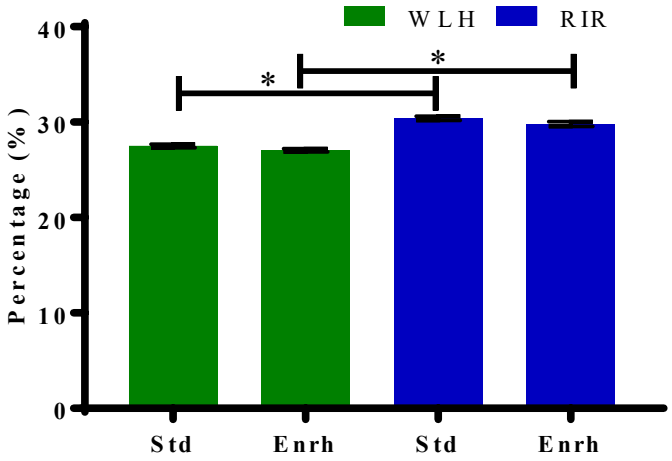

Figure 3. 2. White Leghorn (WLH) and Rhode Island Red (RIR) yolk percentage from standard and enriched diets. Error bars represent standard error of the mean (SEM); *statistically significant difference (p<0.05, one-way ANOVA, Tukey test).

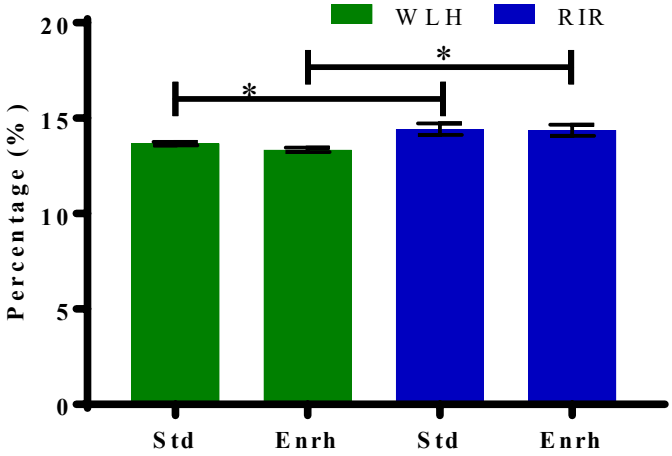

Figure 3.3. White Leghorn (WLH) and Rhode Island Red (RIR) shell percentage from standard and enriched diets. Error bars represent standard error of the mean (SEM); *statistically significant difference (p<0.05, one-way ANOVA, Tukey test).

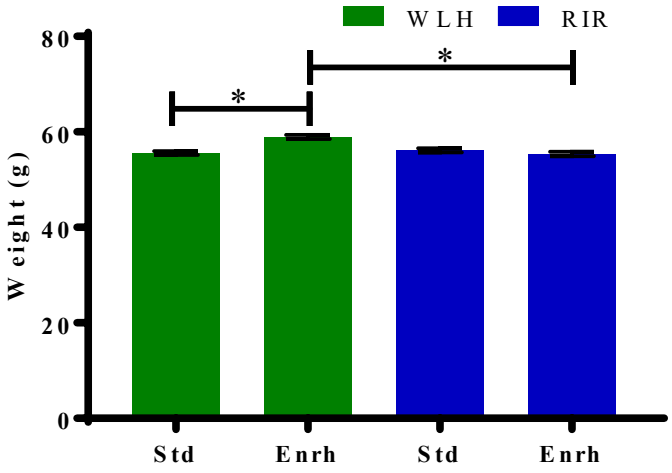

Figure 3.4. White Leghorn (WLH) and Rhode Island Red (RIR) egg weight from standard and enriched diets. Error bars represent standard error of the mean (SEM); *statistically significant difference (p<0.05, one-way ANOVA, Tukey test).

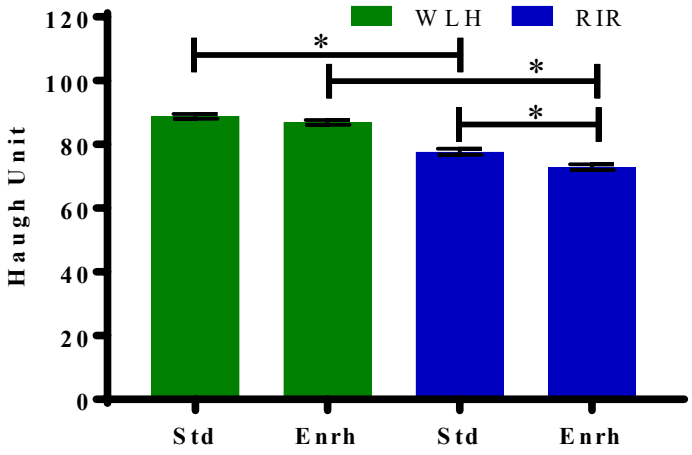

Figure 3.5. White Leghorn (WLH) and Rhode Island Red (RIR) Haugh unit from standard and enriched diets. Error bars represent standard error of the mean (SEM); *statistically significant difference (p<0.05, one-way ANOVA, Tukey test).

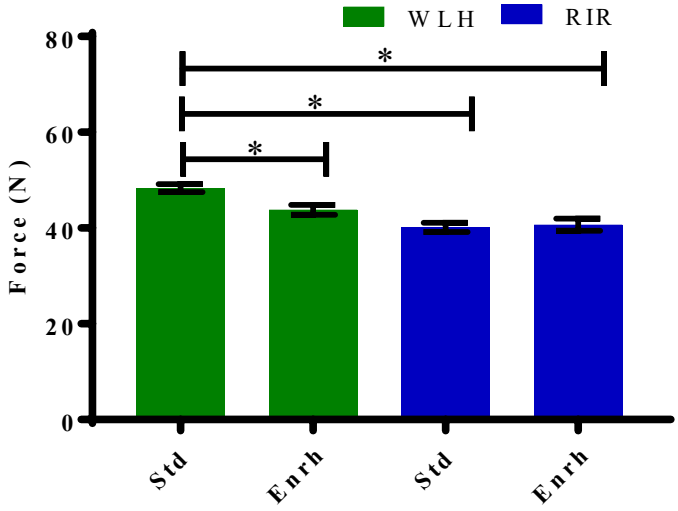

Figure 3.6. White Leghorn (WLH) and Rhode Island Red (RIR) eggshell strength from standard and enriched diets. Error bars represent standard error of the mean (SEM); *statistically significant difference (p<0.05, one-way ANOVA, Tukey test).

CHAPTER 4. CHEMICAL CHARACTERIZATION AND BIOACTIVITY OF COOKED STANDARD AND ENRICHED WHOLE EGGS FROM WHITE LEGHORN AND RHODE ISLAND RED

Abstract

The eggs role in human nutrition is projected to increase in years to come as an animal protein source rich in nutrients. Such nutritional value is derived from its essential amino acids, lipids, vitamins, and minerals is enhanced with bioactive molecules enrichment such as omega-3 fatty acids and carotenoids. Nonetheless, little is known about how domestic cooking may modulate the biological effect of egg components in the human diet. Eggs from White Leghorn (WLH) and Rhode Island Red (RIR) hen breeds were fed with a corn-soybean meal-based diet and a corn-soybean meal-based diet supplemented with flaxseed and ORO GLO® as a carotenoid extract. Analysis of polyunsaturated fatty acid composition and carotenoid content confirmed the enrichment of linolenic and docosahexaenoic fatty acids and lutein in enriched eggs. Egg digestibility was assessed through the degree of hydrolysis quantified by the pH-Stat method, which results showed that eggs were equally digestible independent of its production and cooking methods. However, the hydrolysates peptide content and the biological activity was affected by all three factors. The oxygen radical antioxidant capacity (ORAC) analyzed the antioxidant activity and showed WLH capacity was higher when fried while RIR capacity was higher when boiled. The angiotensin-converting enzyme (ACE) inhibition determined the hydrolysate capacity to inhibit the enzyme, showing RIR boiled had the highest ACE inhibitory activity. No inhibition of the ECE-1 enzyme was observed from the hydrolysates. The peptide profile through a reverse-phase (RP)- and

hydrophilic interaction chromatography (HILIC)-mass spectrometry (MS/MS) showed no difference in peptide size, but a higher intensity for the peptides derived from WLH fried samples. Therefore, whole egg biological activity is dependent on its production and cooking process. Further research is needed to understand the mechanisms which yield such bioactivities.

4.1. Introduction

The egg is known for its nutritional quality with high availability in high- and middle-income countries (Morris, Beesabathuni, & Headey, 2018). Despite the controversial debate of cholesterol content in egg and its effect on the human body, its consumption is projected to increase up to 2030 in most regions of the world (Morris et al., 2018). This wide reach converts the egg into a high impact food. Such characteristics make the egg an important part of human nutrition. Efforts to increase the egg nutritional value have been successful. Compounds such as polyunsaturated fatty acids, carotenoids, trace minerals, and lipid- and water-soluble vitamins were incorporated with success (Chung, Rasmussen, & Johnson, 2004; Coorey, Novinda, Williams, & Jayasena, 2015; Schiavone & Barroeta, 2011). Benefits from incorporating these compounds, such as lutein, into the egg can be observed in a higher bioavailability in human subjects when compared to plant sources (Chung et al., 2004).

The incorporation of bioactive molecules is a strategy to produce functional foods. These products have been shown to enhance health and reduce the risk of diseases (Shahidi, 2004). Such benefits would, in turn, lessen the burden on national health systems. Recent studies have shown the effectiveness and cost-effectiveness of plant sterol-enriched functional foods as a strategy for reducing the risk of cardiovascular

diseases (CVD) (W. Yang, Gage, Jackson, & Raats, 2018). Enriched eggs are a food product containing nutrients with health-beneficial properties and could also help in the prevention of CVD.

Another approach involves the utilization of the natural components of foods to enhance their functional role. In this case, proteins have been shown to contain bioactive peptides. Processes to free these peptides from their parent proteins include microwaves, high hydrostatic pressure, high-intensity ultrasound, fermentation, enzymatic hydrolysis, among others (Dávalos, Miguel, Bartolomé, & López-Fandiño, 2004; Nakamura et al., 1995; Ozuna, Paniagua-Martínez, Castaño-Tostado, Ozimek, & Amaya-Llano, 2015). Additionally, domestic and natural processes such as cooking and gastrointestinal digestion naturally hydrolyze proteins into amino acids and peptides. Previous studies showed how cooking and simulated gastrointestinal digestion modulate the egg biological activities (Jahandideh et al., 2014; Li & Shah, 2004; Majumder & Wu, 2009; Nimalaratne, Savard, Gauthier, Schieber, & Wu, 2015; Remanan & Wu, 2014).

Nonetheless, little is known as how the hen's genetic background, egg nutrient enrichment, and cooking after gastrointestinal digestion modulate its biological activity. Understanding the role of these factors is vital in improving the egg's role as a functional food. Therefore, the purpose of this study is to evaluate the effect of hen genetic, egg enrichment, and cooking methods on egg digestibility and to evaluate the bioactivity of cooked whole egg hydrolysate through *in vitro* methods.

4.2. Materials and Methods

4.2.1. Materials and sample collection

Fresh White Leghorn (white shell) and Rhode Island Red (brown shell) eggs were collected from the Poultry Building F at the University of Nebraska-Lincoln. The hens were 37 weeks old at the beginning of the trial. The hens were fed with a standard diet and an enriched diet containing flaxseed and ORO GLO®[2] as a source of omega-3, lutein, and zeaxanthin for 16 weeks. The collected eggs were stored at 4°C until further preparation without exceeding 21 days.

4.2.2. Preparation of boiled and fried whole egg

Fresh whole eggs were boiled by placing the eggs in a saucepan with boiling water for 10 min; the water covered the egg up to 1 inch above the eggshell. The boiling water was removed, and the boiled egg cooled by running tap water for 5 min. The egg was peeled and stored in a vacuum bag at -80°C until further analysis. Fried whole eggs were prepared by homogenizing the egg yolk and egg white with a home whisker. Once homogenized, the egg was poured into a pan fryer heated at 191°C (375°F) and cooked for 90 s per side. The fried egg was cooled at room temperature for 15 min and stored as the boiled eggs. The cooked eggs were protected from light during storage at 4°C until further analysis.

4.2.3. Simulated gastrointestinal digestion

The simulated gastrointestinal digestion followed Minekus et al. (2014) protocol with Mat et al. (2018) modifications (Mat, Cattenoz, Souchon, Michon, & Le Feunteun,

[2] ORO GLO® was provided by Kemin Corporation.

2018; Minekus et al., 2014). A Titrando 902 pH-stat coupled with an 800 Dosino device (Metrohm AG, Herisau, Switzerland) was used to record the pH and the volume of titrand used to adjust the pH throughout the reaction time of the gastric and intestinal phases. Eggs were thawed overnight at 4 °C and posteriorly dried by absorbing the thawed water. A manual mincer (Kitchen Basics, ASIN: B00JX0ENHE) simulated the mouth mechanical chewing. In the oral phase, ten grams of the minced cooked egg was weighed with an analytical balance and transferred into a stomacher bag (Seward; BA6040/CLR). Afterward, 9 mL of simulated salivary fluid (SSF) with 1 mL of α-amylase (750 U/mL) both tempered at 37°C was added and stomached for 2 min with a Seward Stomacher® 80. The gastric phase started by transferring the oral bolus into a 100 mL jacketed beaker and adding 16.8 mL of simulated gastric fluid (SGF), followed by a pH adjustment to three. Later, 3.2 mL of gastric porcine (25,000 U/mL) solution was added to start the digestion and maintained during 2 h at 37°C. The end of the gastric phase followed the addition of 30 mL of simulated intestinal fluid (SIF), 5 mL of bile salts (10 mM concentration in final volume), and a pH adjustment to 7. The addition of 10 mL of a pancreatin solution (800 U/mL; based on protease activity) started the intestinal digestion and was maintained during 2 h at 37°C. A pH reduction to six inactivated the pancreatin enzymes; the solution is frozen at -80°C for posterior freeze-drying. The process was realized under dim light to avoid photooxidation of egg compounds. Finally, the degree of hydrolysis (DH) is determined by the pH-stat method calculation based on the following equations (Mat et al., 2018):

Equation 4.1. Degree of hydrolysis of gastric digestion.

$$DH_{gastric} = 100 \times \frac{V\ (HCl)\ \times M(HCl)}{m\ (protein) \times\ h_{tot}} \times \frac{1}{(1 - \alpha\ (COOH))} \quad [1]$$

Equation 4.2. Degree of hydrolysis of intestinal digestion.

$$DH_{intestinal} = 100 \times \frac{V\ (NaOH)\ \times M(NaOH)}{m\ (protein) \times\ h_{tot}} \times \frac{1}{\alpha(NH_2)} \quad [2]$$

Where V is the titrand volume (mL), M the molarity of the titrand (M), m is the protein mass (g), h_{tot} is the number of peptide bond per gram of protein (7.67 for egg), α (COOH) the mean degree of dissociation of carboxylic acid (0.09), and $\alpha(NH_2)$ the mean degree of dissociation for the amino groups (0.44) (Mat et al., 2018). An average of the gastric and intestinal digestion is calculated to report the final DH. The simulated gastrointestinal digestion is done in triplicate.

4.2.4. Hydrolysate fatty acid extraction

Whole egg hydrolysate lipid extraction followed the Folch et al. (1957) method with modifications (Folch, Lees, & Sloane Stanley, 1957). An amount of 250 mg of hydrolysate is weighed into 25 mL Erlenmeyer flasks, followed by the addition of 5 mL of chloroform/methanol (2:1 w/v). A rotary shaker shakes the capped Erlenmeyer for 15 min. The solution is transferred to a 15 mL polypropylene tube and centrifuged at 1693 *xg* for 15 min at 4 °C (Thermo Fisher Sorvall Legend X1R, Waltham, MA). The supernatant is collected and transferred to a 15 mL polypropylene tube while adding 1 mL of double distilled water with 0.09% of sodium chloride (NaCl) and vortexed for 15 s. Centrifugation at 188 *xg* for 10 min at 4 °C separated the water-methanol phase from the chloroform. The lower chloroform layer is collected, transferred into glass amber

vials, and the solvent evaporated using a nitrogen flush at room temperature. The sample is stored at -80°C until fatty acid methylation analysis. Extractions are done in duplicate.

4.2.5. Fatty acid methylation and polyunsaturated fatty acid profile

Fatty acid methyl esters (FAME) preparation of hydrolysate lipid extracts was performed according to Belayneh et al. (2015) (Belayneh, Wehling, Cahoon, & Ciftci, 2015; J. Yang & Ciftci, 2017). The fatty acid methylation reaction took place in a screw-cap test tube. The test tube contained 5 mg of lipid extract. Afterwards, 1.5 mL of 2.5% (v/v) sulfuric acid in methanol (containing 0.01% butylated hydroxyl toluene, BHT), 400 µL of toluene, and 200 µL of triheptadecanoin solution (10 mg/mL) was added to each test tube. The tube thread had a double layer of polytetrafluoroethylene (PTFE) sealant tape. The tube was flushed with nitrogen, capped, vortexed, and heated at 90 °C for 1.5 h. Once cooled, 1 mL of water and 1.5 mL of heptane is added to each tube and vortexed. The solution is transferred to a 15 mL tube and centrifuged at 3643 *xg* for 5 min at 25°C (Thermo Fisher Sorvall Legend X1R, Waltham, MA). The supernatant heptane layer is collected and transferred into 1 mL amber glass vials. The samples were stored at -20°C until further analysis. A gas chromatograph (GC) (7890A GC systems, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a flame ionization detector (FID) analyzed the fatty acid methyl esters (FAME). An Agilent HP-INNOWAX capillary column (30 m × 0.25 mm × 0.25 mm) separated the FAME. The instrument held the oven temperature at 90°C for 1 min, then heated it to 235°C at 10°C/min, and then kept it at 235°C for 5 min. The carrier gas used was hydrogen. Fatty acids were identified by comparison of their retention times with those of authentic standards, and the results were reported as the area percentage of the peaks (Belayneh et al., 2015).

4.2.6. Carotenoid analysis

This work was done by the Proteomics and Metabolomics Facility at the Nebraska Center for Biotechnology at UNL. The following carotenoids, Lutein and Zeaxanthin were extracted according to Dautermann and Lohr, 2017. Briefly, an aliquot of the egg powder was extracted with 100% acetone and homogenized for 10 min at 20 Hz using the Tissue Lyser II (Qiagen). After centrifugation at 16,000 g, the supernatants were collected and transferred into an HPLC vial (Dautermann & Lohr, 2017).

For LC separation, Acclaim C30 column (2.1 mm × 150 mm, 3 μm, Dionex) was used flowing at 0.3 mL/min at 30°C on a UPLC Infinity II (Agilent) equipped with a DAD. The gradient of the mobile phases A (Acetonitrile/Methanol, 2:1, v/v) and B (Methanol/Ethyl Acetate, 1:1, v/v) was as follow: 100% A for 2 min, to 95% A in 6 min, to 85% A in 2 min, hold at 85% A for 4 min, to 100% A in 0.5 min. The carotenoids were detected using DAD at 440 nm. For quantification, an external standard curve was prepared using a series of standard samples containing different concentrations of carotenoids mixture.

4.2.7. Sample preparation and peptide estimation

The sample preparation for the peptide estimation and *in vitro* bioactivity analyses was as follows: 1 mg/mL hydrolysate solution was prepared with double distilled water in a 15 mL test tube and vortexed for 1 min. Consequently, the solution was centrifuged at 4600 x*g* for 10 min at 4 °C and filtered with a 0.45 μm nylon syringe filter. The Pierce™ Quantitative Fluorometric Peptide Assay (23290, Thermo Scientific, Waltham, MA) was used to quantify the peptide concentration in the whole egg hydrolysate. The sample solution was diluted up to 125 μg/mL with double distilled water, and the peptide

quantified according to the manufacturer's instructions. The result was adjusted by the sample weight and expressed as peptide concentration (%). The sample preparation was done in duplicate and assayed in duplicate.

4.2.8. Measurement of antioxidant activity

The oxygen radical absorbance capacity (ORAC) method measured whole egg hydrolysate antioxidant activity. The hydrolysate hydrophilic and hydrophobic extract preparation and their antioxidant assay followed Remanan and Wu (2014) and Nimalaratne et al. (2011) methodology (Nimalaratne, Lopes-Lutz, Schieber, & Wu, 2011; Remanan & Wu, 2014). A 50 mL Erlenmeyer flask contained 50 mg of hydrolysate. Next, 10 mL of hexane-dichloromethane (1:1) was added to each flask and shaken in an orbital shaker for 1 h at 25°C, followed by centrifugation at 1693 x*g* for 5 min at 4°C (Thermo Fisher Sorvall Legend X1R, Waltham, MA). The supernatant is collected and evaporated under nitrogen flush, obtaining the lipophilic extract. Consequently, a nitrogen flush dried the pellet, later dissolved in 10 mL 80% ethanol, and vortexed. The hydrophilic extraction process repeated the steps of the hydrophobic extraction process. The supernatant was used for the hydrophilic ORAC analysis. The lipophilic extract was dissolved using 250 µL of acetone and 750 µL of 7% Randomly Methylated Beta Cyclodextrin (RMCD) in 50% acetone-water solution (1:1; v/v). RMCD acted as a solubility enhancer for the lipid fraction. It was also used to dissolve the Trolox standards and in the blank. All samples are extracted in duplicate and assayed in triplicate.

4.2.9. Measurement of ACE inhibitory activity

The ACE Kit-WST (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) measured the whole egg hydrolysate ACE inhibitory activity (Hai Bang et al., 2014). A

whole egg hydrolysate solution was prepared according to section 2.7 with several dilutions (8, 40, 200, 500, 800, 1000 µg/mL). The sample solution was mixed with the ACE substrate 3HB-GGG (3-hydroxybutyrate glycylglycylglycine) and the ACE enzyme in a 96 well plate followed by incubation at 37°C for 1 h. In this step, the substrate was cleaved into 3HB-G and G-G and then into 3HB and G. Afterwards, an indicator working solution was added to each well and incubated at room temperature for 10 min. The indicator solution used 3HB to reduce the tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)- 2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt (WST-1) through a chain reaction into formazan. The ACE-inhibition was measured indirectly by the formazan concentration at 450 nm (Hai Bang et al., 2014; Lam, Shimamura, Manabe, Ishiyama, & Ukeda, 2008). The regression analysis determined the ACE inhibition IC_{50} value for each sample. The assay was performed in duplicate with two independent assays per sample.

4.2.10. Measurement of vasodilation capacity

The ECE-1 inhibitory capacity of whole egg hydrolysates followed Gallego et al. (2019) method with modifications (Fernández-Musoles et al., 2010; Gallego, Mora, Hayes, Reig, & Toldrá, 2019). A 2 mg/mL hydrolysate solution was prepared with 100 mm 4-morpholineethanesulfonic acid buffer (pH 6, MES) buffer and vortexed for 1 min. Consequently, the solution was centrifuged at 4600 *xg* for 10 min at 4°C and filtered with a 0.45 µm nylon syringe filter. Next, 14 µL of sample at different concentrations (0.01, 0.1, 0.5, 1, 1.5 mg/mL) was mixed with 110 µL of a 0.220 µg/mL ECE-1 solution in 100 mM MES buffer (pH 6) containing 150 mM NaCl. Finally, the solution was mixed with 72 µL 35 µM Mca-R-P-P-G-F-S-A-F-K(Dnp)-OH Fluorogenic Peptide Substrate V (FPS

V) in 100 mM MES buffer containing 150 mM NaCl (pH 6) and incubated at 37°C for 20 min. A Synergy H1 microplate reader (Biotek, Winooski, USA) read the reaction fluorescence at 355 nm excitation and 405 nm emission at the beginning and the end of the assay. The IC_{50} was calculated for each sample. The assay was performed in duplicate with two independent assays per sample.

4.2.11. Identification of the peptide profile through UPLC-MS/MS

The whole egg hydrolysate <3000 Da fraction was collected through ultracentrifugation (Catalog # UFC9003, Millipore Sigma, Burlington, MA) and freeze-dried for further analysis. This work was done by the Proteomics and Metabolomics Facility at the Nebraska Center for Biotechnology at UNL. An aliquot of the dried samples was resuspended in water at a concentration of 20 µg/µL. For the HILIC separation of the peptides with 2-3 amino acids, the samples were further diluted 10 times for a 10 µg injection. The separation of the peptides was done on a BEH-Amide 1.7 µm (2.1 x 100 mm, Waters) using a Vanquish (Thermo) HPLC at 40°C and at a flow rate of 300 µL/min with a gradient of A (0.1% formic acid in 100% LC-MS grade water) and B (0.1% formic acid in 100% acetonitrile) as follow: 90% B for 2 min, 90% to 40% B in 12 min, back to 90% in 1 min . The data was acquired on a QE-HF (Thermo) mass spectrometer using a mass range of 60 to 750 m/z on single charged ions. The isolated ions were further fragmented using isolation window of 2 m/z. The acquired data was analyzed using PEAKS studio (Bioinformatics Solutions Inc., Waterloo, Canada) to perform de novo sequencing of the chromatograms and integration of the peaks for quantification.

For the separation of the larger peptides, samples were diluted 200 times and run using an online peptide separation by first desalting peptides on a trapping column (C18 Pepmap100 0.3x5mm, 5µm, 100A) at 5µL/min in 1% acetonitrile, 0.1% formic acid before separation into the mass spectrometer using a 75µm x 25cm peptide CSH C18 130A, 1.7µm nano-column (Waters) using a linear gradient run at 260 nL/min from 5% B to 32% B over 36min. Solvents: A is 0.1% formic acid in LC-MS grade water, and B is 0.1% formic acid in 80% acetonitrile. The Q Exactive HF was run in a data-dependent acquisition mode triggering on peptides with charge states 1 to 4 over the mass range of 375-1500 m/z. All MS/MS samples were analyzed using PEAKS studio (Bioinformatics Solutions Inc., Waterloo, Canada). PEAKS studio was set up to search the UniProt-ref_prot_Gallus_gallus_UP000000539 database (20190617, 27804 entries) assuming the digestion enzyme no specific. Peptides were searched with a fragment ion mass tolerance of 0.020 Da and a parent ion error tolerance of 5.0 ppm.

The data was exported to Excel. The peptides sequences were subjected to structural requirement constrains of antioxidant peptides. The constraints were the presence of Tyr/Trp or Leu/Phe/Ile in the amino terminal with the presence of Lys, Leu, His, Trp, Met, Tyr, and Glu in any position at the carboxylic end (Aluko, 2015; Dávalos et al., 2004; Y. F. Liu, Oey, Bremer, Carne, & Silcock, 2018; Zhang, Xiong, Chen, & Zhou, 2013). Both conditions needed to be met to be counted as a potential peptide with antioxidant activity. Similarly, the peptides sequences were subjected to structural requirement constrains of ACE-inhibitory di-, tri- and oligopeptides as described by Wu et al. (2006)(Jianping Wu, Aluko, & Nakai, 2006b, 2006a). The peptide needed to comply with all the constrains to be counted as a potential ACE-inhibitory peptide. No

quality cutoff such as ALC for *de novo* analysis or -10logP for UniProt search was used in the data analysis. The hydrophilic interaction chromatography (HILIC) and Reverse Phase (RP) chromatography data were analyze separately.

4.2.12. Statistical analysis

The statistical analysis was performed through a one-way ANOVA comparison between treatments involving different hen breed, diet, and cooking methods for simulated gastrointestinal digestion degree of hydrolysis. Significant differences were determined using the Tukey test at the level of $p<0.05$. A two-tailed unpaired *t-test* determined the statistical difference for the ACE inhibitory capacity activity measurement through ACE-inhibition. Significant differences were determined at the level of $p<0.05$. A two-way ANOVA analysis determined the statistical difference for the sections of polyunsaturated fatty acid profile, carotenoid analysis, hydrolysate peptide estimation, antioxidant activity through ORAC assay, and vasodilation capacity through ECE-1 inhibitory activity. Significant differences were determined using the Bonferroni's test at the level of $p<0.05$ (Dunn, 1961). The data are expressed as mean ± standard error of the mean (SEM).

4.3. Results and Discussion

4.3.1. White Leghorn (WLH) and Rhode Island Red (RIR) whole egg omega-3 enrichment

The whole eggs obtained from WLH and RIR hens fed with standard and enriched diets for 100 days showed a statistical difference in omega-3 content within each treatment as shown in Figure 4.1. The means and *p*-values can also be observed in the supplementary data section on Table 4.3. The enriched samples had a higher amount

of linolenic acid (18:3) in all the treatments compared to their standard counterparts. The linolenic acid present in the standard samples was no longer observed after the samples were subjected to a gastrointestinal digestion. Arachidonic acid as shown in Figure 4.1B was significantly reduced for the enriched samples in all the treatments. Reducing the content of omega-6 fatty acids in the whole egg. Figure 4.1C shows the content of docosahexaenoic fatty acids which was significantly higher for the enriched samples compared to their standard counterparts in all the treatments. These results are in accordance with the previous research of egg enrichment with polyunsaturated fatty acids through the feed of the hen (Coorey et al., 2015; Lemahieu et al., 2015; Nowacki et al., 2017). Additionally, Ahn et al. 1995 found no difference between WLH and RIR variability on enriched eggs linolenic, arachidonic, and docosahexaenoic fatty acids similar to our results (Ahn, Sunwoo, Wolfe, & Sim, 1995). No significant difference was found between cooked samples and their digested counterparts through a two-way ANOVA applying the Bonferroni's test. Malondialdehyde has been measured in cooked eggs as an indicator of lipid oxidation with similar values between normal, omega-3/lutein enriched, Heritage brown leghorn, and Heritage white leghorn eggs. The study found that the hen breed did not have an effect on the lipid peroxidation under oxidizing conditions (Nimalaratne, Schieber, & Wu, 2016). Additionally, studies analyzing the egg fatty acid composition found not significant differences of the fatty acid composition when subjected to hard-boiled and scrambled cooking methods (Cortinas, Galobart, Barroeta, Baucells, & Grashorn, 2003). Another study did not saw a significant difference between polyunsaturated fatty acids in cooking methods unless an external source of fat was added to the cooking process (Douny et al., 2015). No significant decrease of the

fatty acid content was observed after digesting the fried and boiled samples of each hen breed in our study. Fatty acid production on gastrointestinal digestion is through gastric and pancreatic lipases, which absorb to emulsified lipids and convert triacylglycerols to monoacylglycerols and fatty acids. The digestion products are later solubilized within mixed micelles and vesicles that transport them to epithelium cells through mucous later (Giang et al., 2016; Hoffman & Borgstrom, 1964; Hur, Lim, Decker, & McClements, 2011). Therefore, the fatty acid content is expected to remain stable after gastrointestinal digestion until cellular uptake.

4.3.2. White Leghorn (WLH) and Rhode Island Red (RIR) whole egg carotenoid enrichment

As shown in figure 4.2, the lutein content was significantly higher between standard and enriched samples for WLH-B, WLH-F, RIR-B, RIR-F, and RIR-F-D. Additionally, significant differences through the process were found for enriched samples between RIR-B vs. RIR-B-D (33.29 vs. 17.3, $p<0.0002$) and RIR-F vs. RIR-F-D (33.78 vs. 23.35, $p<0.0267$) in which the lutein content was lower after gastrointestinal digestion. The means and *p*-values can also be observed in the supplementary data section on Table 4.4. In contrast, this process effect was not significant between WLH-B vs. WLH-B-D and WLH-F vs. WLH-F-D. Therefore, the lutein content was not reduced significantly after digestion for WLH, while RIR did have a significant reduction. No difference was found for standard egg lutein content after gastrointestinal digestion. The results under this experiment suggest that even though enrichment is achieved on the egg, the bioaccessibility is on the same level as standard eggs except for RIR-F-D. Previous studies had shown the xanthophyll's stability of standard eggs under gastrointestinal

digestion through a TIM-1 gastrointestinal model. Lutein and zeaxanthin from boiled, scrambled, and fried eggs were stable during a standard egg gastrointestinal digestion (Nimalaratne, Savard, et al., 2015). The lutein stability in the standard egg is in accordance with our results from WLH.

No significant differences were found for zeaxanthin in any treatment through a two-way ANOVA applying the Bonferroni's test. The results obtained in our study where on a range between 4 to 5 µg/g of whole egg hydrolysate for the standard and enriched eggs. Therefore, the enrichment on zeaxanthin was not achieved. Previous reports had shown that lutein enrichment decreases the content of zeaxanthin on egg yolk (Leeson & Caston, 2004). Recent studies showed that frying and scrambled cooking treatments resulted in a bioaccessibility reduction of zeaxanthin, but remain stable trough *in vitro* digestion (Nimalaratne et al., 2016). Even though no reduction was seen between the cooking treatments, the zeaxanthin was stable to gastrointestinal digestion as no difference was observed between the cooked and digested samples.

4.3.3. Degree of hydrolysis of cooked whole egg

Cooked whole egg subjected to boiling and frying methods showed a DH range of 25-28%, as shown in Table 4.1. These values are higher compared to previous research on crude egg white, in which a 2 h digestion obtained a 9.4% DH (Chen, Chi, Zhao, & Xu, 2012). The effect of the cooking method and its relationship with the degree of hydrolysis and peptide production has also been reported by Sangsawad et al. (2017). A heat treatment at 121°C and 15 psi for 1 h showed the lowest DH while a heat treatment of 70°C during 30 min showed the highest DH for proteins of chicken breast. Therefore, intense heat treatment induces protein crosslinking, aggregation, sulfoxidation,

carbonylation, and hydroxylation resulting in a lower digestibility (Sangsawad, Roytrakul, & Yongsawatdigul, 2017). Based on previous research, it is expected that exposing food to a mild heat treatment increases its DH during the simulated gastrointestinal (GI) digestion. Even though the cooking methods did not show any difference in digestibility, this parameter could influence the biological activity of the hydrolysates as a mild heat treatment could have less amino acid oxidation and smaller peptides.

4.3.4. Peptide content of cooked whole egg hydrolysates

When subjected to a boiling process, no statistical difference was found between WLH and RIR irrespective of diet, as shown in Figure 4.3A. However, Figure 4.3B shows that RIR enriched fried samples showed a lower peptide content than WLH when enriched. Possibly suggesting a hen line breed and enrichment effect, which modulates the peptide content in the hydrolysates. Our previous data showed a higher albumen percentage for WLH eggs and a higher yolk percentage for RIR eggs (section 3.1.2). Egg white proteins account for a higher proportion of whole egg proteins (Kovacs-Nolan, Phillips, & Mine, 2005). Therefore, it could influence the WLH higher peptide content. Additionally, Wang et al. (2011) showed a higher egg white protein abundance on RIR eggs for ovalbumin, ovoglycoprotein, ovalbumin-related protein Y, ovomucoid, and prostaglandin D_2 synthase (Wang, Liang, Omana, Kav, & Wu, 2012). The abundance of ovomucoid, a heat-stable serine protease inhibitor, could reduce the peptide release in RIR fried samples (Kovacs-Nolan et al., 2005; Nolasco, Guha, & Majumder, 2019; Shuichi, Noriko, Atsuko, Izumi, & Kin-ichiro, 1994). It is unknown to which extent such a result is an effect of storage as ovomucoid degrades during prolonged storage times (M.

Liu, Yu, Ren, & Wu, 2018). Figure 4.3C represents the effect between enrichment and cooking method for WLH samples, which did not show a statistical difference. Nevertheless, RIR showed a higher peptide content when boiled rather than fried on enriched samples (Figure 4.3D). As previously mentioned, mild heat treatment favors the release of peptides from its parent protein (Sangsawad et al., 2017). This is seen for RIR boiled samples when compared to its fried counterpart.

4.3.5. Antioxidant capacity of whole egg hydrolysates through Oxygen Radical Absorbance Capacity (ORAC)

Figure 4.4A represents boiled WLH and RIR whole egg hydrolysates, which did not show any statistical difference in its antioxidant activity for standard or enriched samples. The frying process showed that WLH standard and enriched eggs had a higher antioxidant capacity than its RIR counterparts (Figure 4.4B). Even though no direct comparison was made, Heritage White Leghorn showed a slightly higher ORAC value than Heritage Brown Leghorn when fried (77.1 and 62.7 μmol TEAC/g sample, respectively), showing a similar trend as our data (Nimalaratne et al., 2016). It is noted that the frying process decreased the antioxidant capacity of RIR standard and enriched hydrolysates, as well as the WLH enriched samples (Figure 4.4C and 4.4D). It is proposed that this reduction is seen due to the high heat treatment during frying, responsible for degradation, isomerization, or oxidation of antioxidants such as carotenoids (Nimalaratne et al., 2016). Additionally, it has been reported that heat treatment reduces aromatic amino acids from egg yolk, responsible for its high antioxidant activity (Nimalaratne et al., 2011). Finally, the highest antioxidant capacities observed were WLH standard fried and RIR boiled samples irrespective of the diet.

4.3.6. ACE inhibition capacity of whole egg hydrolysates

The sample with the highest ACE inhibitory activity (lowest IC_{50}) was observed in RIR boiled with no statistical difference between standard and enriched (IC_{50}: 127 and 117 µg/mL, respectively), as shown in Figure 4.5B. Suggesting a synergistic effect between the antioxidant and ACE inhibitory activity of RIR boiled egg. Additionally, WLH boiled enriched sample had a higher ACE inhibitory activity when compared to the standard egg (Figure 4.5A). Similarly, WLH and RIR fried enriched samples tended to a higher ACE-inhibitory activity. These samples are expected to have short peptides, a characteristic often found on ACE-inhibitory peptide (Y. F. Liu et al., 2018). The mechanism by which these peptides are obtained in the enriched samples remains to be elucidated. However, it is proposed that the carotenoids and omega-3 in enriched samples interact to a higher degree with the lipid fraction of the sample, leaving the protein fraction accessible for hydrolysis.

4.3.7. ECE-1 inhibition capacity of whole egg hydrolysates

The samples of whole egg hydrolysate were found to have no ECE-1 inhibition capacity as all samples were found to have inhibition values above 100%. Figure 4.6 represents the IC_{50} value obtained from the reaction being a higher value better as more protein is required to promote the ECE-1 activity. This lack of inhibition has previously been shown from peptides derived from lactoferricin (Fernández-Musoles et al., 2010). Previous studies showed that FPS V is selective but not specific for ECE. Therefore, Big-ET1 yields better results since it is an ECE-1 natural substrate (Fernández-Musoles et al., 2010, 2013). However, a limitation of the assay is the crossreactivity of the endothelin EIA kit (Cayman Chemicals, Ann Arbor, MI, USA) with Big-ET1, ET-1, ET-

2, and ET-3 (100% in all) might result in nonspecific signal. An alternative substrate or an alternative method should be further studied to determine ECE-1 inhibition capacity *in vitro*.

4.3.8. Whole egg hydrolysate LC-MS/MS peptide profile

The result shown in Figure 4.7 indicates a higher number of peptides within the seven to eight amino acids (AA) residues. Samples WLH fried and RIR fried standard along with WLH boiled enriched had a higher number of peptides with 5 AA residues. Also, the distribution of the peptide intensity is higher in the five AA residues for each sample, as shown in Figure 4.8. The sample with the highest intensity of peptides in the range of three to 11 amino acid residues is WLH fried standard. Several studies have reported the antioxidant activity of peptides once released from their parent protein (Dávalos et al., 2004; Nimalaratne, Bandara, & Wu, 2015; Wang, Liao, Nimalaratne, Chakrabarti, & Wu, 2018). We assume the results are linked to the antioxidant activity as WLH fried standard showed the highest ORAC value.

Figure 4.9 describes the number of hits and intensity of the peptides, which complied with the antioxidant activity structural requirements. The samples with the highest intensity in Figure 4.9B had a higher concentration of peptides that complied to the antioxidant activity structural requirement. WLH fried standard showed a higher intensity of peptides with this property. Therefore, the antioxidant activity observed in ORAC is assumed to be due to the structure of the peptide along with its concentration. In contrast, the peptide profile of RIR boiled standard and enriched samples, which showed the highest antioxidant capacity *in vitro* did not show a high intensity. It is proposed that the antioxidant activity of the samples is due to the synergistic effects of

peptides in the hydrolysate, although further research is required to determine such an effect.

The structural requirement constrains for ACE-inhibitory peptides were the following: dipeptides required amino acid residues with bulky and hydrophobic side chains as Phe, Tyr, or Trp in both positions, tripeptides favored aromatic amino acid for the carboxyl terminus, positively charged amino acids in the middle residue and hydrophobic amino acids for the amino terminus. Tetrapeptides favored Tyr, Phe, or Cys in the carboxyl terminus, Phe in the second residue from the carboxyl terminus, Arg, His, Trp, or Phe in the third residue, and Val, Ile, or Trp in the fourth residue. Similarly, oligopeptides favored Tyr or Cys in the carboxyl terminus, His, Trp, or Met in the second residue from the carboxyl terminus, Ile, Leu, Val, or Met in the third residue, and Trp in the fourth residue. It was observed that only dipeptides complied with all the constraints from the literature. However, di- and tripeptides with previously reported ACE-inhibitory activity the were found in the samples, as shown in Table 4.2. These peptides comply in part with the determined constraints. It is assumed, based on these results that peptides having at least two of the mentioned structural characteristics could potentially have ACE-inhibitory activity. A recent study using quantitative-structure relationship (QSAR) model built with an artificial neural network showed that the C-terminal is of primary importance for ACE-inhibitory dipeptides when containing a hydrophobic amino acid (He et al., 2012). As shown in Table 4.2, the dipeptides which did not complied to both structural requirements did have a hydrophobic and aromatic amino acid in the C-terminal, supporting our assumption.

4.4. Concluding Remarks

The effect of hen genetic, egg nutrient enrichment, and cooking method showed it modulated the whole egg peptide content and its bioactivity. The eggs from hens fed with the enriched diet contained a higher amount of linolenic and DHA fatty acids in cooked and digested samples with no effect from hen genetics and cooking methods. Similarly, eggs from hens fed with the enriched diet had a higher content of lutein after being cooked, but not after digestion with content similar to standard eggs. Enriched eggs did not have higher levels of zeaxanthin when compared to standard eggs. Additionally, standard eggs lutein and zeaxanthin levels did not decrease after being subjected to cooking methods or simulated gastrointestinal digestion. Such information is of importance to address the bioaccessibility of bioactive molecules from enriched eggs in the human digestive system.

Hen genetics, egg enrichment, and cooking method did not affect the egg protein digestibility. The peptide content differed as an effect of hen genetics and enrichment, with a higher peptide content for WLH fried enriched and RIR boiled enriched. The peptide content suggested a partial contribution to the antioxidant activity as RIR boiled enriched eggs showed high antioxidant activity. Consequently, enriched samples tended to have a better inhibitory activity, with a significant difference for WLB boiled enriched. Egg from RIR boiled samples showed the best ACE inhibitory activity irrespective of diet, showing a synergistic effect with its antioxidant activity. The peptide profile through RP- and HILIC-LC-MS/MS showed differences in the peptide size intensity due to a cooking effect and potential bioactive peptides based on structural requirements. Therefore, whole egg biological activity is dependent on its production and cooking

process. Further research is needed to understand the mechanisms which yield such bioactivities.

4.5. References

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Tables

Table 4.1. Degree of hydrolysis (DH, %) of cooked whole egg hydrolysates.

Cooking method	White Leghorn		Rhode Island Red	
	Standard	Enriched	Standard	Enriched
Boiled	27.21 ± 0.91	28.10 ± 2.35	27.56 ± 1.54	27.16 ± 2.04
Fried	25.55 ± 0.96	25.17 ± 1.13	28.12 ± 0.69	26.56 ± 0.68

Note: No statistical difference among the treatments.

Table 4.2. Potential ACE Inhibitory peptides intensity present in the whole egg hydrolysates from whole egg and egg white proteins analyzed through RP and HILIC LC-MS/MS.

Peptide sequence	WLH Boiled Enriched	RIR Boiled Standard	RIR Boiled Enriched	WLH Fried Standard	RIR Fried Standard	WLH Fried Enriched	Source	Enzyme used	Reference
FF	5.68E+06	5.88E+06		9.78E+06	7.74E+06	7.91E+06	Whole egg	Pepsin-Pancreatin	
YY			2.36E+06	4.59E+06		4.51E+06	Whole egg	Pepsin-Pancreatin	
FY		4.53E+06	3.49E+06		3.16E+06		Whole egg	Pepsin-Pancreatin	
VRFP					1.48E+06		Whole egg	Pepsin-Pancreatin	
LW	2.01E+07	1.43E+07		1.19E+07	1.04E+07	1.30E+07	Ovalbumin	Pepsin	(Vendramini-Costa & Carvalho, 2012)
LY			6.41E+07	7.41E+07			Ovotransferrin	Chymotrypsin	(Majumder & Wu, 2010)
NF		3.07E+06	5.09E+06	2.86E+06	3.25E+06	2.19E+06	Ovalbumin	Pepsin-Pancreatin	
YR	2.54E+06	2.32E+06	1.13E+06	1.32E+06	1.64E+06	1.54E+06	Ovalbumin	Pepsin-Pancreatin	(Grootaert et al., 2017)
AW		1.46E+06	1.64E+06		1.34E+06		Lysozyme	Chymotrypsin-Thermolysin	
MPF	4.69E+07	4.14E+07	9.30E+07	6.43E+07	5.12E+07	4.48E+07	Ovotransferrin	Pepsin-Pancreatin	

Note: Di- and tripeptides analyzed through *de novo* sequencing. Tetrapeptides analyzed through UniProt database comparison.

Table 4.2. continued

Peptide sequence	WLH Boiled Enriched	RIR Boiled Standard	RIR Boiled Enriched	WLH Fried Standard	RIR Fried Standard	WLH Fried Enriched	Source	Enzyme used	Reference
ADHP		3.22E+06	7.62E+06		6.62E+06	6.75E+06	Ovalbumin	Pepsin-Pancreatin	(Miguel, Alvarez, López-Fandiño, Alonso, & Salaices, 2007)
Total	5.51E+07	6.19E+07	1.78E+08	1.57E+08	7.64E+07	6.77E+07			

Note: Di- and tripeptides analyzed through *de novo* sequencing. Tetrapeptides analyzed through UniProt database comparison.

Figures

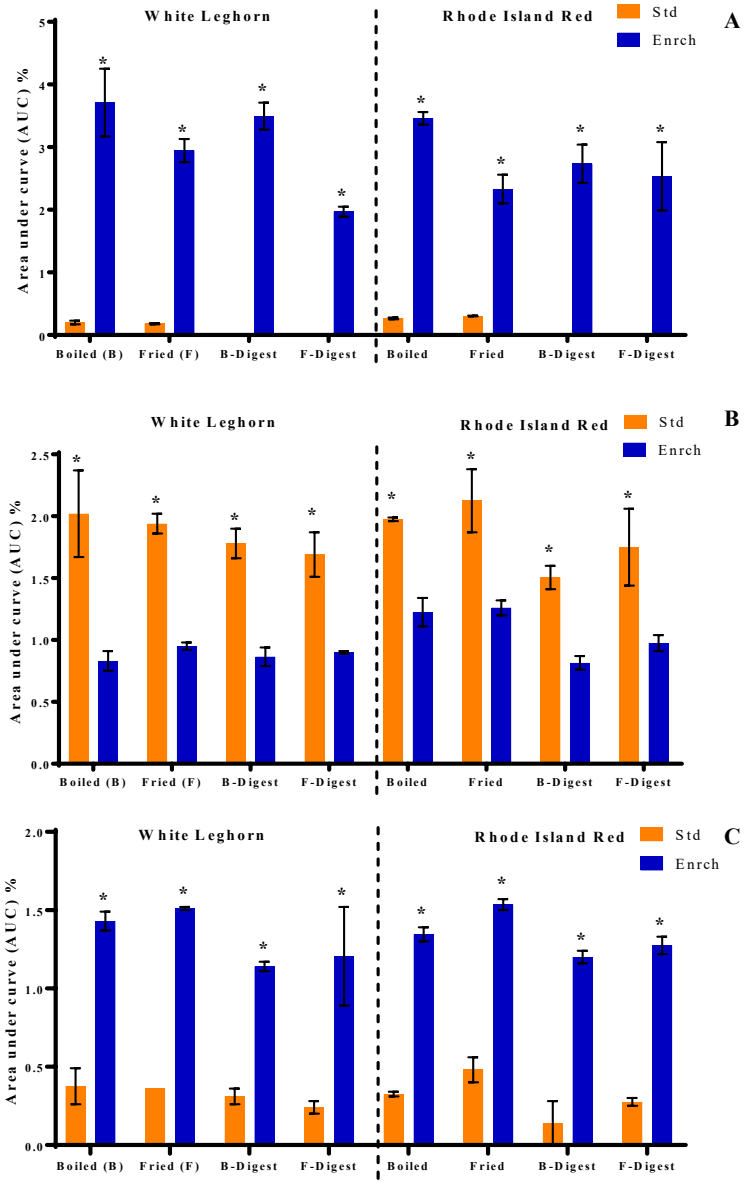

Figure 4.1. Linolenic (C18:3)(A), arachidonic (C20:4)(B), and docosahexaenoic (C22:6)(C) fatty acid composition (% area under the curve (AUC)) in cooked and digested whole egg samples. Note: Left bar: standard, right bar: enriched. B: boiled, F: fried, D: digested. Error bars represent standard error of the mean (SEM); *statistically significant difference (p<0.05, two-way ANOVA, Bonferroni's test).

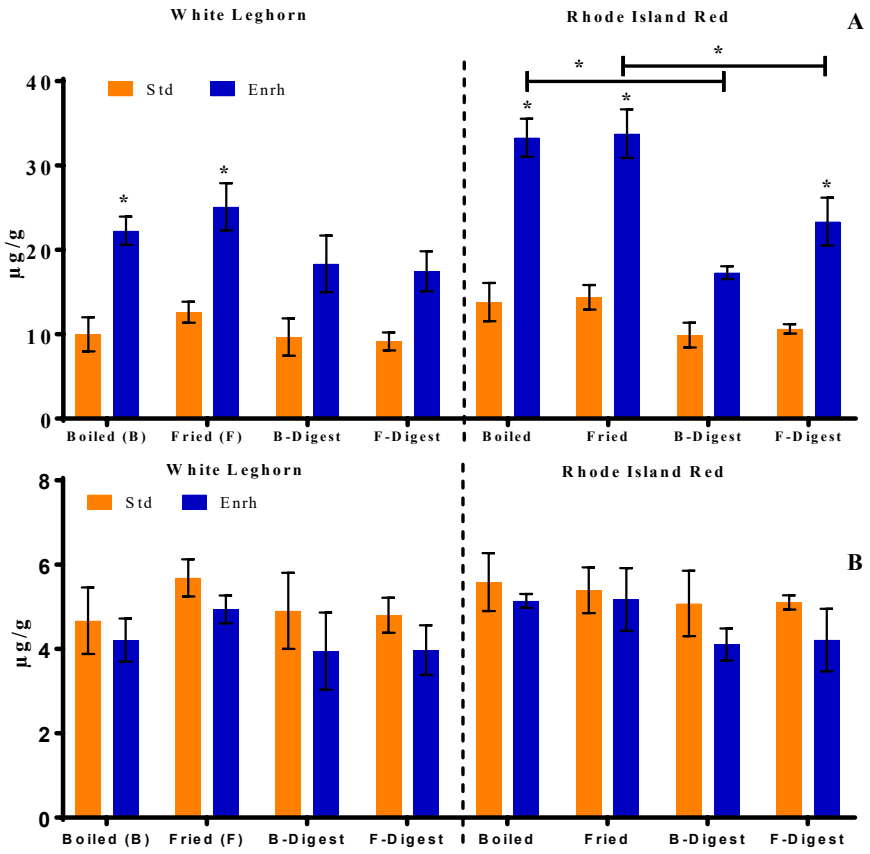

Figure 4.2. Lutein (A) and zeaxanthin (B) content (µg/g of hydrolysate) incooked and digested whole egg samples. Note: Left bar: standard, right bar: enriched. B: boiled, F: fried, D: digested. Error bars represent standard error of the mean (SEM); *statistically significant difference ($p<0.05$, two-way ANOVA, Bonferroni's test).

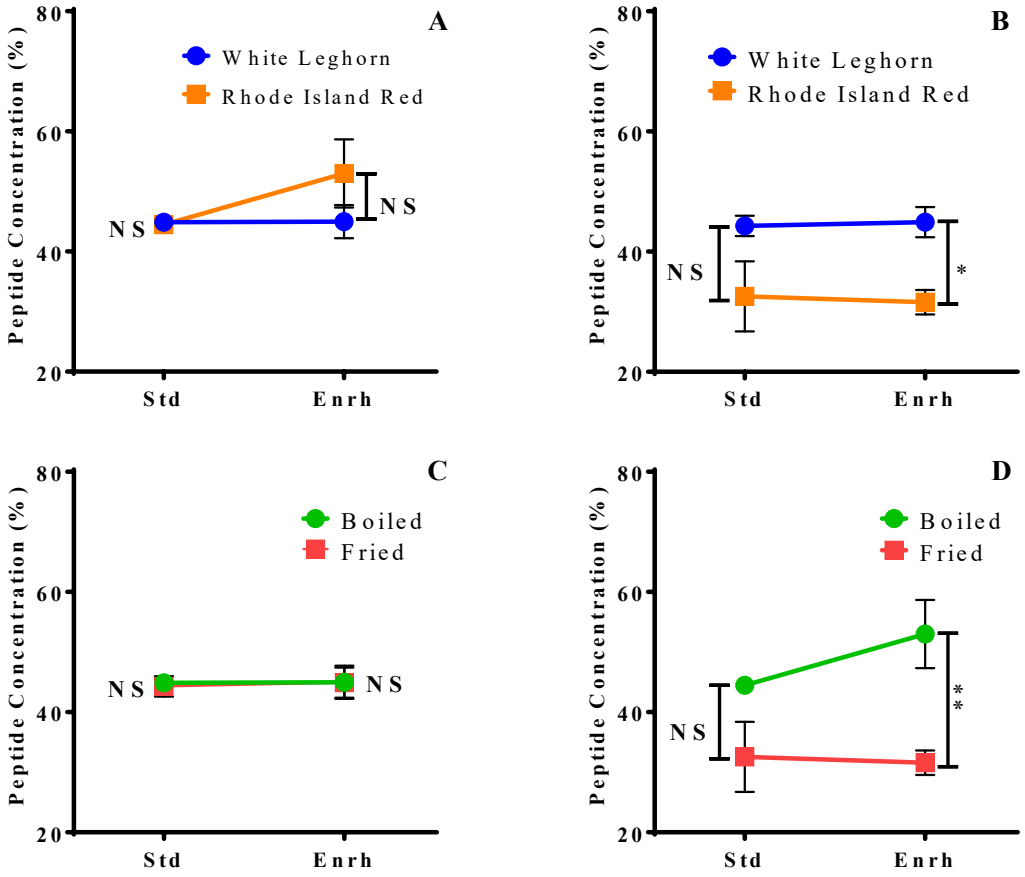

Figure 4.3. Peptide concentration of WLH and RIR whole egg hydrolysate; Error bars represent standard error of the mean (SEM); *statistically significant difference (p<0.05, two-way ANOVA, Bonferroni's test). Note: A: boiled; B: fried; C: WLH; D: RIR.

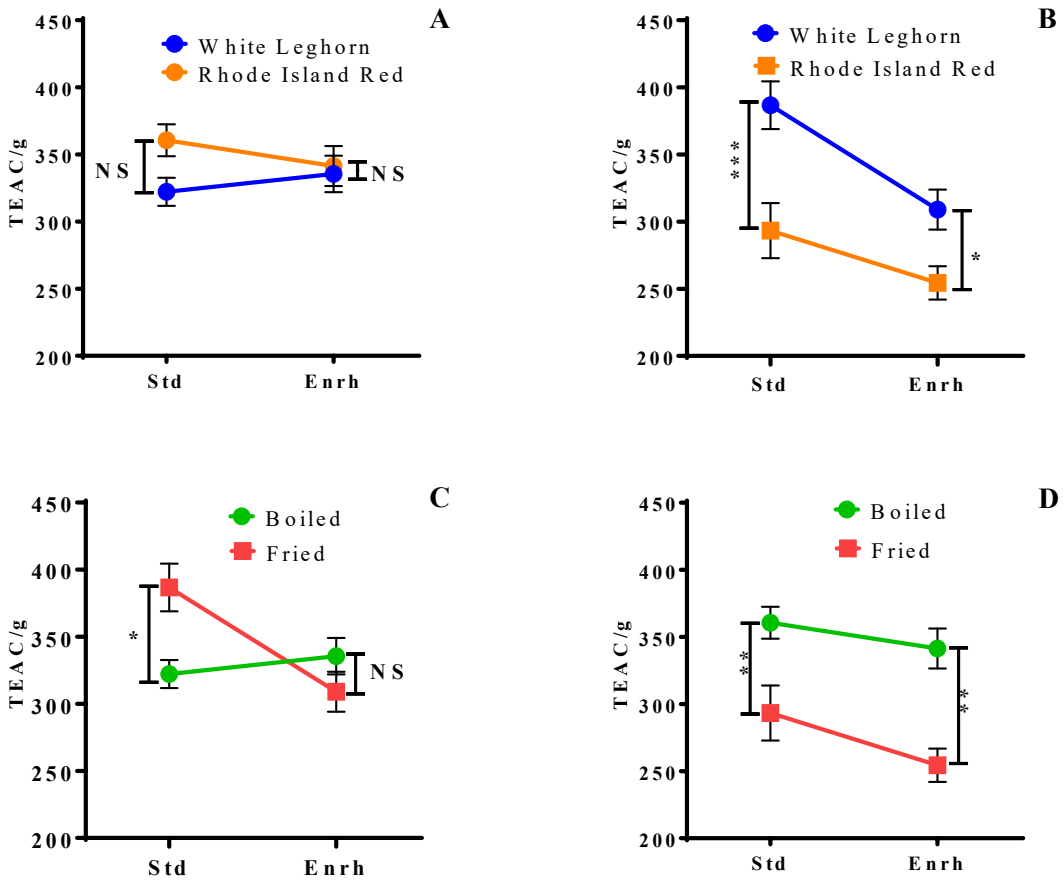

Figure 4.4. Total ORAC of whole egg hydrophilic and hydrophobic extract. Error bars represent standard error of the mean (SEM); *statistically significant difference (p<0.05, two-way ANOVA, Bonferroni's test). NS: Nonsignificant. Note: Trolox Equivalent Antioxidant Capacity (TEAC/g of hydrolysate); A: boiled; B: fried; C: WLH; D: RIR.

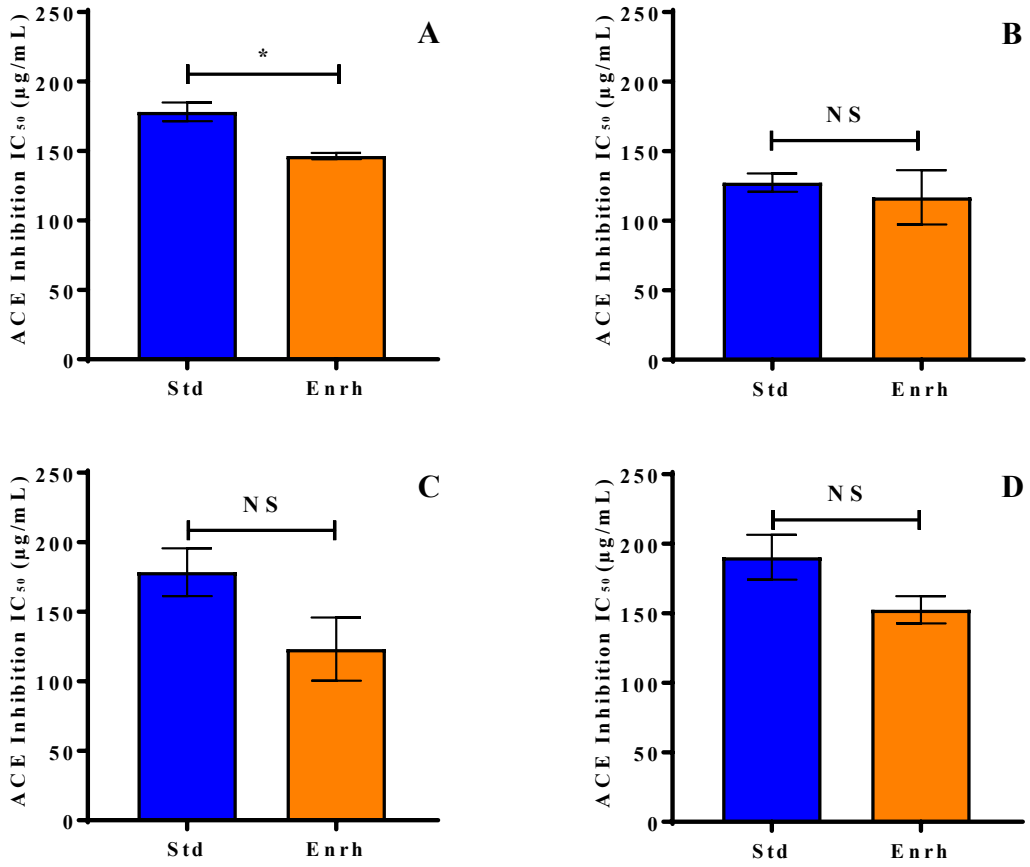

Figure 4.5. Whole egg hydrolysates ACE Inhibition IC_{50}; Error bars represent standard error of the mean (SEM); *Statistically significant difference (p<0.05); NS: Nonsignificant. Note: IC_{50}: Protein concentration which inhibits 50% of ACE activity; A: WLH boiled; B: RIR boiled; C: WLH fried; D: RIR fried.

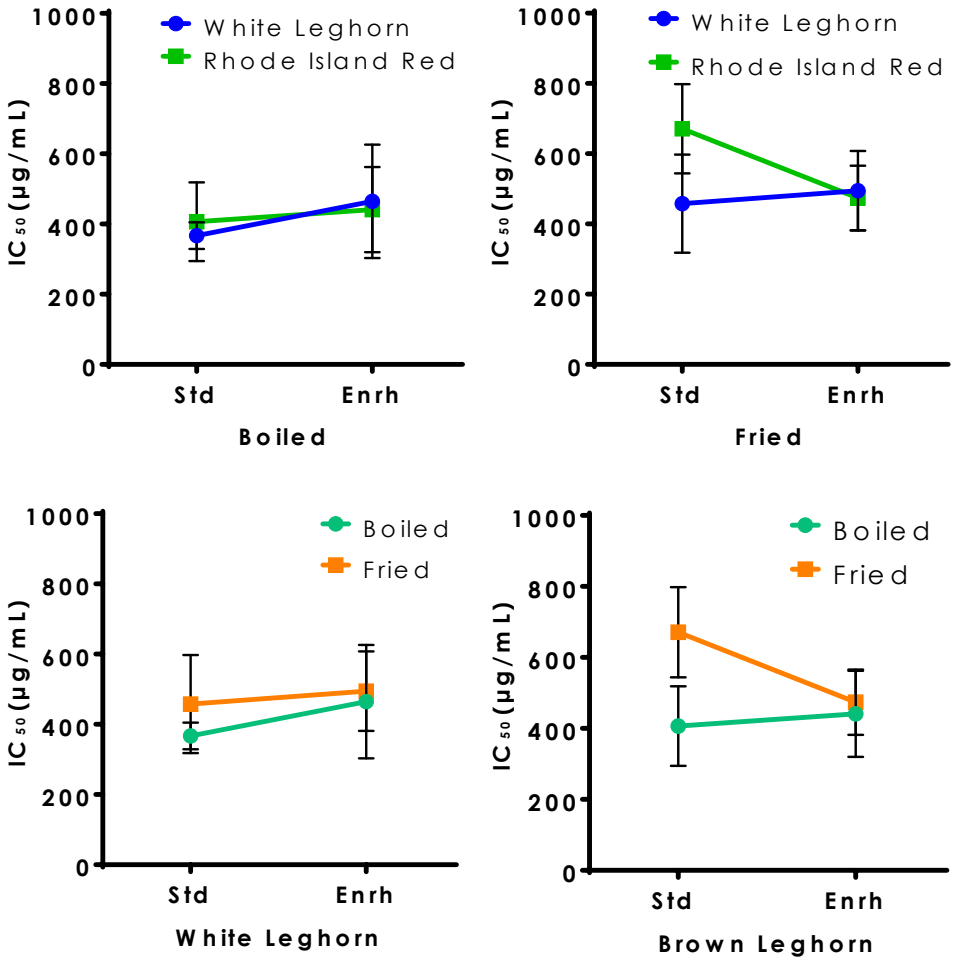

Figure 4.6. ECE-1 *in vitro* promoting activity of whole egg hydrolysate. A: boiled, B: fried, C: WLH, and D: RIR. Note: Error bars represent standard error of the mean (SEM); *statistically significant difference (p<0.05, two-way ANOVA, Bonferroni's test). No significant difference was found between the treatments.

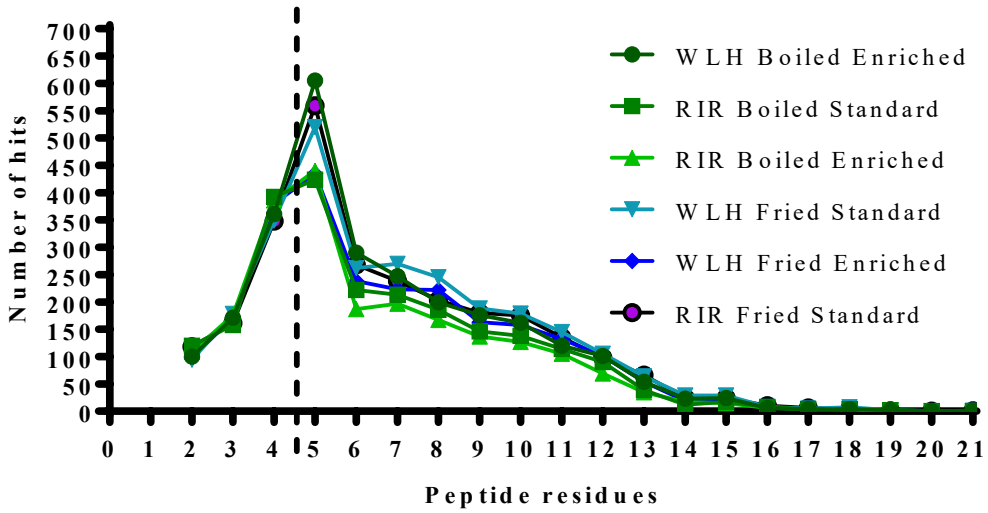

Figure 4.7. Peptide size distribution by peptide residues measured through RP and HILIC LC-MS/MS. Note: Peptide length from two to four amino acid residues were anlayzed through PEAKS studio *de novo* sequencing. Peptide with five and more residues were analyzed through PEAKS studio through UniProt database.

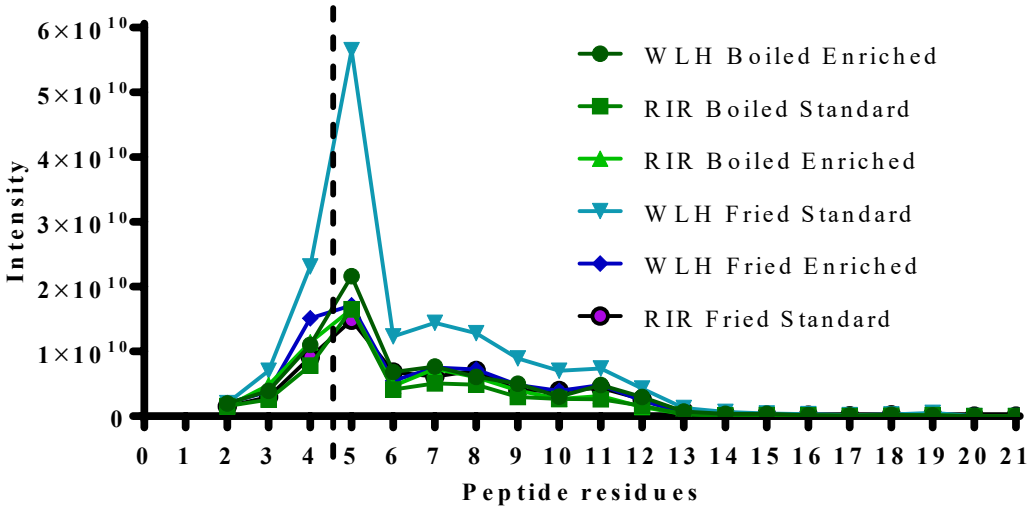

Figure 4.8. Peptide size intensity distribution measured through RP and HILIC LC-MS/MS. Note: Peptide length from two to four amino acid residues were anlayzed through PEAKS studio *de novo* sequencing. Peptide with five and more residues were analyzed through PEAKS studio through UniProt database.

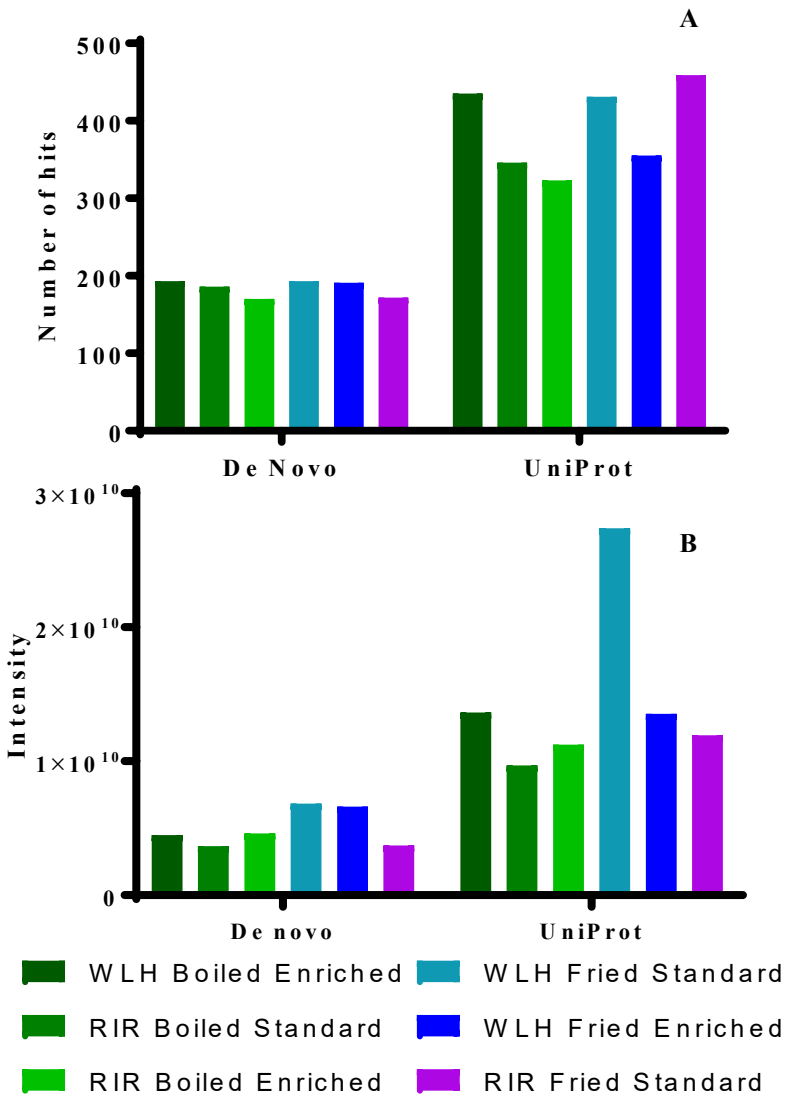

Figure 4.9. Number of hits and intensity of whole egg peptides with potential antioxidant activity based structural requirements. Note: *De novo* include peptides with two to four AA residues. Uniprot include peptides with five or more AA residues.

Supplementary data

Table 4.3. Comparison between standard and enriched cooked whole egg and whole egg hydrolysate fatty acid composition area under the curve (AUC).

Description-enrichment effect	Mean±SEM 1 (%)	Mean±SEM 2 (%)	*p*-value
Linolenic acid (C18:3)			
WLH boiled (B) std (1) vs WLH boiled enrh (2)	0.20±0.03	3.71±0.54	<0.0001
WLH fried (F) std vs WLH fried enrh	0.18±0.01	2.95±0.19	<0.0001
WLH B-digest std vs WLH B-digest enrh	0.00±0.00	3.50±0.22	<0.0001
WLH F-digest std vs WLH F-digest enrh	0.00±0.00	1.97±0.08	0.0001
RIR B std vs RIR B enrh	0.27±0.01	3.46±0.10	<0.0001
RIR F std vs RIR F enrh	0.31±0.01	2.33±0.23	<0.0001
RIR B-digest std vs RIR B-digest enrh	0.00±0.00	2.74±0.31	<0.0001
RIR F-digest std vs RIR F-digest enrh	0.00±0.00	2.54±0.55	<0.0001
Arachidonic acid (C20:4)			
WLH boiled (B) std (1) vs WLH boiled enrh (2)	2.02±0.35	0.83±0.08	0.0004
WLH fried (F) std vs WLH fried enrh	1.94±0.08	0.95±0.03	0.0028
WLH B-digest std vs WLH B-digest enrh	1.78±0.12	0.87±0.08	0.0056
WLH F-digest std vs WLH F-digest enrh	1.69±0.18	0.90±0.01	0.0188
RIR B std vs RIR B enrh	1.97±0.01	1.23±0.12	0.0277
RIR F std vs RIR F enrh	2.13±0.26	1.26±0.06	0.0091
RIR B-digest std vs RIR B-digest enrh	1.51±0.10	0.82±0.06	0.0493
RIR F-digest std vs RIR F-digest enrh	1.75±0.31	0.98±0.07	0.0217
Docosahexaenoic acid (C22:6)			
WLH boiled (B) std (1) vs WLH boiled enrh (2)	0.38±0.12	1.43±0.06	<0.0001
WLH fried (F) std vs WLH fried enrh	0.36±0.00	1.51±0.01	<0.0001
WLH B-digest std vs WLH B-digest enrh	0.31±0.05	1.14±0.03	0.0002

WLH F-digest std vs WLH F-digest enrh	0.24±0.04	1.21±0.32	<0.0001
RIR B std vs RIR B enrh	0.33±0.02	1.35±0.05	<0.0001
RIR F std vs RIR F enrh	0.48±0.08	1.54±0.04	<0.0001
RIR B-digest std vs RIR B-digest enrh	0.14±0.14	1.20±0.04	<0.0001
RIR F-digest std vs RIR F-digest enrh	0.28±0.03	1.28±0.06	<0.0001

Note: Note: SEM: standard error of the mean, WLH: White Leghorn, RIR: Rhode Island Red, Std: standard, Enrh: enriched.

Table 4.4. Comparison between standard and enriched cooked whole egg and whole egg hydrolysate carotenoid content (µg/g of hydrolysate).

Description-enrichment effect	Mean±SEM 1 (%)	Mean±SEM 2 (%)	*p*-value
	Lutein		
WLH boiled (B) std (1) vs WLH boiled enrh (2)	9.987±2.03	22.26±1.67	0.0020
WLH fried (F) std vs WLH fried enrh	12.61±1.24	25.11±2.79	0.0016
WLH B-digest std vs WLH B-digest enrh	9.67±2.21	18.34±3.36	0.0518
WLH F-digest std vs WLH F-digest enrh	9.15±1.07	17.46±2.36	0.0699
RIR B std vs RIR B enrh	13.81±2.27	33.29±2.26	<0.0001
RIR F std vs RIR F enrh	14.38±1.46	33.78±2.87	<0.0001
RIR B-digest std vs RIR B-digest enrh	9.903±1.46	17.30±0.75	0.1465
RIR F-digest std vs RIR F-digest enrh	10.63±0.55	23.35±2.84	0.0013
	Zeaxanthin		
WLH boiled (B) std (1) vs WLH boiled enrh (2)	4.67±0.78	4.21±0.51	>0.9999
WLH fried (F) std vs WLH fried enrh	5.68±0.44	4.94±0.33	>0.9999
WLH B-digest std vs WLH B-digest enrh	4.90±0.90	3.95±0.92	>0.9999
WLH F-digest std vs WLH F-digest enrh	4.80±0.42	3.97±0.59	>0.9999
RIR B std vs RIR B enrh	5.59±0.69	5.14±0.17	>0.9999
RIR F std vs RIR F enrh	5.39±0.54	5.17±0.75	>0.9999
RIR B-digest std vs RIR B-digest enrh	5.08±0.77	4.11±0.38	>0.9999

RIR F-digest std vs RIR F-digest enrh	5.10±0.17	4.21±0.74	>0.9999

Note: Note: SEM: standard error of the mean, WLH: White Leghorn, RIR: Rhode Island Red, Std: standard, Enrh: enriched.

CHAPTER 5. OVERALL CONCLUSION

Egg nutritional characteristics involve complex but promising implications in human health. Additionally, the egg enrichment potentiates it as a functional food. Previous egg enrichment research focuses on the enrichment effect on egg quality. The hens can deliver specific compounds to the egg, maintaining its quality. However, the effect of hen breed, enrichment, and cooking method effect on egg nutrients digestibility and bioaccessibility are poorly understood. The results obtained in this dissertation are expected to reduce the knowledge gaps on the function of whole egg domestic consumption as a functional food. Both factors, hen breed and diets, maintained the overall egg quality with differences in egg components percentages and a higher Haugh unit in WLH eggs. Such results confirm the capacity of the poultry industry to produce high-quality eggs with added value from bioactive compounds such as omega-3 fatty acids and carotenoids.

Further studies addressed the effect of boiled and pan-frying cooking methods on standard and enriched egg digestibility and biological activities. Higher content of omega-3 fatty acids and lutein in enriched eggs confirmed both hens were able to deliver bioactive molecules. The enrichment did not affect the digestibility of the whole egg. However, the cooking methods and the hen breed did affect the peptide content. Such information elucidate how egg from different hen breeds behave under different cooking methods. This information helps the industry to recommend suitable ways of domestic preparation for egg products depending on its production.

Similarly, the bioactivity of the whole egg was modulated based on its hen breed, enrichment, and cooking methods. The antioxidant activity was modulated by hen breed,

enrichment, and cooking methods. It was observed that WLH eggs had higher antioxidant activity than RIR when fried. Moreover, the standard WLH fried egg had higher antioxidant activity than its enriched counterpart. When comparing RIR between boiled and fried, the boiled samples had higher antioxidant activity than the fried counterpart irrespective of diet.

Consequently, the RIR samples showed a synergistic activity as their ACE inhibitory activity was the highest for the boiled samples irrespective of diet. WLH boiled enriched samples also showed a high ACE inhibitory activity, while the WLH fried enriched egg showed the highest ACE-inhibitory activity for the fried samples. Such results suggest that egg enrichment is an alternative to potentiate the egg ACE inhibitory activity derived from egg peptides.

The *in vitro* biological activity is partly complemented with the RP- and HILIC LC-MS/MS peptide profile. It is assume the antioxidant and ACE inhibitory activity from the hydrolysates is based on the peptide size, intensity, and structural requirements. Its proposed that a synergistic effect can take place and enhance the hydrolysates biological activities. Further research is necessary to confirm the biological activity of the new whole egg peptides by chemical synthesis and activity evaluation.

Finally, this study is a continuous effort to understand how the production and processing of the egg and possibly other foods can modulate their biological activity. It also suggests that consumers can obtain benefits from bioactive food compounds in reducing the risk factors associated with cardiovascular diseases. Further research should be performed on *in vitro* cell studies and *in vivo* models to confirm the effects of these functional foods.