CHARACTERIZATION OF THE MAJOR ANTIMICROBIAL COMPONENTS OF THE CHILEAN TINAMOU *(NOTHOPROCTA PERDICARIA)* EGG WHITE

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ABSTRACT

Chilean tinamou (*Nothoprocta perdicaria*) egg white was characterized and compared to chicken (*Gallus gallus*) and emu (*Dromaius novaehollandiae*) egg whites for composition and antimicrobial components. Tinamou and chicken appeared more similar in terms of protein, sialic acid, ash, and iron content than tinamou and emu, even though phylogenetic analysis places tinamous within the ratites.

Egg white proteins were separated by anion–exchange Fast Protein Liquid Chromatography, followed by SDS–PAGE. Tinamou ovotransferrin, ovomucoid, and lysozyme C were identified by peptide mass fingerprinting of SDS-PAGE bands. Similar sized ovotransferrins were present in all egg whites species, however higher quantities were observed for ratites. It is possible that ovotransferrin has an essential antimicrobial function and therefore its presence is conserved among distinct species.

The antimicrobial activity of tinamou and chicken ovotransferrins against two food related pathogens, *Escherichia coli* O157:H7 and *Staphylococcus aureus* COL was bicarbonate, concentration, and avian species dependent, as evaluated by turbidly and viability assays. Native ovotransferrins were the most effective against *E. coli* O157:H7, followed by apo and holo forms. Native ovotransferrins exhibited a significant bactericidal activity at a concentration of 10 mg/ml with bicarbonate. In the presence of bicarbonate, chicken apo and holo ovotransferrins were more bacteriostatic than tinamou ovotransferrins. Additionally, there was no significant difference in the antimicrobial activity of apo and holo ovotransferrins applied at 5 and 10 mg/ml. Holo ovotransferrins exhibited moderate antimicrobial activity, only in the presence of bicarbonate; therefore it is possible that bicarbonate contributes to the antimicrobial activity of ovotransferrin by a mechanism other than a bridging ligand between ovotransferrin and iron. Native chicken and tinamou ovotransferrins at 10 mg/ml were bactericidal against *S. aureus* COL, whereas tinamou ovotransferrin was more bacteriostatic.

In conclusion, tinamou ovotransferrin combined with bicarbonate was found to be bactericidal against two foodborne pathogens. In the absence of bicarbonate, tinamou ovotransferrins exhibited minor bacteriostatic activity, while chicken ovotransferrin was not effective. It is possible that

tinamou ovotransferrin possesses different amino acid sequences from the chicken protein that form unique antimicrobial motifs; therefore it should be further investigated as a natural antimicrobial agent for use in food matrices or food preparation surfaces.

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LIST OF ABBREVIATIONS

Abs	Absorbance
BSA	Bovine serum albumin
BHI	Brain heart infusion
CEW	Chicken egg white
CFU	Colony forming units
CM	Carboxymethyl
DDT	Dithiothreitol
DW	Deionized water
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EEW	Emu egg white
EW	Egg white
EWP	Egg white proteins
FPLC	Fast protein liquid chromatography
FRA	Fraction
ICP MS	Inductively coupled plasma mass spectrometry
kDa	kilodalton
Lys	Lysozyme
LB	Luria–bertani
LTQ–FT	Linear ion trap mass spectrometer fourier transform
ML, M. lysodeikticus	*Micrococcus lysodeikticus*
MS	Mass spectrometry
MW	Molecular weight
MDa	Megadalton
NMR	Nuclear magnetic resonance

OD	Optical density
OTAP–92	Ovotransferrin antimicrobial peptide-92
OTF	Ovotransferrin
PNGase-F	Peptide:N–glycosidase F
Rf	Relative mobility
SDS–PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S. aureus	*Staphylococcus aureus*
TEW	Tinamou egg white
Tris–HCl	Tris(hydroxymethyl)aminomethane HCl

ACKNOWLEDGEMENTS

1. INTRODUCTION

Chicken (*Gallus gallus*) eggs are a multi–purpose substance, serving both as a nutritious food source and as a major food processing component. Besides that, chicken eggs are a source of biologically active compounds that can benefit human health. These functional activities include antimicrobial, anti–adhesive, immune–modulatory, anticancer, antihypertensive, antioxidant, and protease inhibition (Mine *et al.*, 2006). These roles emphasize the importance of egg and egg components in human health and in disease control.

Antimicrobial activity is one of the most abundantly studied egg biological properties. This attribute is mostly related to egg white proteins, such as ovotransferrin (OTF), lysozyme, ovomucoid and ovalbumin peptides, and also to a carbohydrate, sialic acid.

Ovotransferrin is one of the major chicken egg white proteins. Ovotransferrin is an iron binding protein that can bind two iron atoms only in the presence of two CO_3^{-2} or HCO_3^{-1} molecules (Schalabach and Bates, 1975). Ovotransferrin is reported to have antibacterial properties against a variety of bacteria (Tranter *et al.*, 1982 and Oratore *et al.*, 1990), among them two foodborne pathogens, *Escherichia coli* O157:H7 and *Staphylococcus aureus*. *E. coli* O157:H7 is a Gram negative pathogen known as Shiga toxin–producing *E. coli,* one of the most common foodborne pathogens. It was first associated as a human pathogen causing outbreaks of bloody diarrhea in the USA in 1982 (Wells *et al.*, 1983; Riley *et al.*, 1983). *S. aureus* COL is a Gram positive Methicillin Resistant (MRSA) bacteria, which produces staphylococcal enterotoxins and is resistant to various antibiotics. It was discovered in the United Kingdom in 1961, and by the mid–1970s had become endemic in many countries (Voss and Doebbeling, 1995). More recently, Lee (2003) reported the presence of MRSA strains in dairy cows and chickens.

Research regarding ovotransferrin antimicrobial activity against these two bacteria has accumulated contradictory results that may be strain dependent. Though there are studies showing that these bacteria are ovotransferrin sensitive, other studies suggest these bacteria are ovotransferrin resistant (Ibrahim *et al.,* 1998; Ko *et al*., 2008; Valenti *et al*., 1980). Additionally, there is a dispute regarding

the mechanism of ovotransferrin resistance: It is suggested that in order to serve as an antimicrobial agent, ovotransferrin should be iron–free (apo–ovotransferrin). Apo–ovotransferrin inhibits bacterial growth by binding essential iron, resulting in iron–saturated ovotransferrin (holo–ovotransferrin) (first recognized by Alderton *et al.*, 1946; Ko *et al.*, 2008). However, other studies suggest that ovotransferrin is antimicrobial regardless of iron saturation state and may be due to membrane disruption (Valenti *et al.,* 1987; Ibrahim *et al.*, 1998 and 2000).

The use of natural antimicrobials has become popular in the food safety industry for they are more acceptable to the public. In some cases, they may be eco–friendly, medically acceptable and economical to manufacture. Due to the dominance of the poultry industry, most research regarding antimicrobial properties of eggs has focused on chicken eggs; therefore it is of interest to find antimicrobial compounds from other avian source, such as tinamous.

Tinamous are ground birds endemic to South America. Recently, phylogenetic analysis has placed the tinamous within the ratites, as a sister group to emus (*Dromaius novaehollandiae*) (Harshmann *et al.*, 2008). Besides that, most of the research regarding tinamous has focused on domestication. An interesting characteristic of the fresh Chilean tinamou (*Nothoprocta perdicaria*) egg white is its distinctive pink hue. It was previously reported that ovotransferrin is pink upon iron saturation (Alderton *et al.*, 1946); therefore Chilean tinamou egg white may serve as an excellent source of this natural antimicrobial agent.

According to this information, it was interesting to investigate the Chilean tinamou egg white proteins and to compare them to chicken and emu egg white proteins, with a specific focus on ovotransferrin, because of its unique antimicrobial activity properties.

The first aim of this study was to identify the major antimicrobial components of the Chilean tinamou (hereinto referred to as "tinamou") egg white and to compare them to the chicken and emu. Since tinamous are a ratite species, the hypothesis was that more similarities will be observed between tinamou and emu egg whites, rather than between tinamou and chicken egg whites. The experimental plan was to compare chicken, emu, and tinamou egg whites antimicrobial components such as

ovotransferrin, lysozyme, ovalbumin, ovomucoid and sialic acid and to perform proteomic analysis to the tinamou egg white. The tinamou egg white proteins were first separated by anion exchange chromatography and gel electrophoresis, following identification by mass spectrometry.

The second aim of this study was to characterize and compare the antimicrobial activity of tinamou ovotransferrin to chicken ovotransferrin against two foodborne pathogens, *E. coli* O157:H7 and *S. aureus* COL. The hypothesis was that tinamou egg white may have high quantities of iron saturated ovotransferrin and therefore can serve as an excellent source of a natural antimicrobial agent. Additionally, it was hypothesised that ovotransferrin is antimicrobial regardless of iron saturation state. The antimicrobial activity of three forms of ovotransferrin from tinamou and chicken were compared – native (as naturally accrues in egg white), apo and holo forms. Ovotransferrin bacteriostatic and bactericidal activities were determined by turbidity and viability assays, respectively, in the presence of bicarbonate.

Characterization of the tinamou egg white proteins and identifying functional proteins is a first step towards potential commercial utilization of these eggs. Evaluating the tinamou ovotransferrin may provide insights into the important protein motifs that are required for antibacterial activity, since the tinamou protein sequence may differ from the chicken sequence.

2. LITERATURE REVIEW

2.1 Chilean tinamou and its egg

The Chilean tinamou is a small ground bird, with grayish brown to olive upper parts, a dark barring and pale streaking. The Chilean tinamou is one of 46 species comprising the tinamou family, widely distributed throughout the Neotropics from Mexico to Patagonia (Withers *et al.*, 1983). The tinamou diet was reported to be essentially granivorous, with invertebrates being consumed in a variable proportion depending of the season (González–Acuñal *et al.*, 2006).

Research on tinamous egg white properties has been conducted in order to determine their taxonomy classification. The results of several biochemical and immunological studies by Osuga and Feeney (1968) on egg white proteins of ratites demonstrated that tinamous were more distantly related to any of the ratites than the ratites were to one another. Prager *et al.* (1976) showed by quantitative immunological comparison of transferrin from ratites, tinamous, and other flying birds that all the ratites and tinamous are of monophyletic origin relative to other birds. Sibley and Ahlquist (1972) have interpreted their starch gel electrophoretic data on the egg white proteins as indicating a close relationship only among the large ratites. Laskowski and Fitch (1989) have found that the amino acid sequences of tinamou ovomucoids are distinct from those of all other birds. The egg white protein electrophoresis analysis has suggested a linkage between tinamous and Galliformes (Caspers *et al.*, 1994), as suggested by Sibley and Ahlquist (1972). Further molecular studies involving DNA–DNA hybridization (Sibley and Ahlquist, 1981), and short DNA sequences (Van Tuinen *et al.*, 2000) agreed on a sister–group relationship of tinamous and ratites. Recently, phylogenetic analyses of 20 unlinked nuclear genes by Harshmann (2008), revealed a genome–wide signal that unequivocally places tinamous within the ratites.

2.2 Emu and its egg

Emu *(Dromaius novaehollandiae)*, the second largest member of the ratite family, has recently became a popular alternative form of livestock world–wide. Emus have a great potential as a valuable

oil and meat source (Berge *et al.*, 1997). The emu egg can serve as a nutritive food source as well, for it is approximately 10 times larger than an extra large chicken egg. An emu egg contains an almost equal volume of yolk and albumen, unlike most avian eggs whose volume of albumen is two times greater than that of the yolk (Koga, 1969; Sales *et al.*, 1996). There has been abundant biochemical research on chicken egg, but significantly few biochemical studies have been performed on emu egg. Recently, Maehashi *et al.* (2010) studied emu egg white and found differences in the composition of the major proteins between emu and chicken egg white. The total protein of the emu egg white was 8.9%. They discovered that unlike the chicken egg white, where ovalbumin is the dominant protein, ovotransferrin is the dominant protein in emu egg white. Additionally, lysozyme and its enzymatic activity were not detected in emu egg white, though they possess the functional genes for both chicken and goose type lysozymes (Maehashi *et al*., 2012).

2.3 Chicken and its egg

Chicken (*Gallus gallus*) is the most common domesticated poultry species, raised worldwide for its meat and eggs for at least 4000 years. The chicken egg has been studied extensively; therefore a wealth of information exists in the literature. The chicken egg is an inexpensive and high nutritional value food source (Mine, 2006). Chicken eggs are comprise of approximately 63% egg white albumen, 27.5% yolk and 9.5% eggshell (including shell membrane) (%w/v). The main components are water (75%), protein (12%), lipid (12%), with minor amounts of carbohydrate and mineral (Li-Chan *et al*., 1995). Most of the proteins are found in the egg yolk and egg white, while a small proportion is present in the egg shell and shell membrane (Sugino *et al.*, 1997). The majority of lipids are found in the egg yolk as lipoproteins. Carbohydrates are a minor egg component, and are present both in free form and attached to proteins and lipids (Sugino *et al.*, 1997). Several minerals, including calcium and magnesium have also been found in eggs, mostly associated with the eggshell.

2.3.1 Chicken egg white antimicrobial components

Eggs have physical and biological defense systems to protect the embryo against the invasion and multiplication of microorganisms. The main line of defense includes the egg shell and shell membrane, which serve mainly as physical barriers. Besides some egg yolk components such as immunoglobulin Y, egg antimicrobial activity has been mostly attributed to egg white components (Li–Chan *et al.*, 1995). The egg white contains various proteins with proven antimicrobial activities that may be related to several mechanisms, including bacterial cell lysis, metal binding, and vitamin binding. The major egg white antimicrobial proteins include lysozyme, ovotransferrin, ovalbumin and ovomucoid. Besides antimicrobial proteins, egg white contains a carbohydrate antimicrobial compound, sialic acid.

2.3.1.1 Lysozyme

Lysozyme is a 14 kDa protein consists of 129 amino acids (Mine, 1995). It is one of the major chicken egg white proteins (3.5% of total egg white proteins) and can be found in the egg white attached to ovomucin, ovotransferrin, and ovalbumin, due to its high isoelectric point of 10.7. Within avian species, two types of lysozymes have been identified, termed lysozyme C and G. The chicken egg lysozyme is typed as C, and it occurs also in geese, swans and ducks. Lysozyme G is more widespread and may be found in tinamou and other ratites species (Prager *et al.*, 1974). Lysozyme G and C were found to differ in molecular weight, amino acid composition, and enzymatic properties, indicating that these two enzymes were the products of two different genes (Arnheim and Steller 1970; Arnheim *et al.*, 1973). With the determination of the three–dimensional structures of C–type (Blake *et al.*, 1965) and G–type (Grutter *et al.*, 1983) lysozymes, it was suggested these lysozymes might have a common evolutionary origin (Grutter *et al.*, 1983; Weaver *et al.*, 1985).

Lysozyme antimicrobial activity has been attributed mostly to two mechanisms, enzymatic hydrolysis and membrane disruption. Its bacteriolytic enzymatic activity involves hydrolysis of ß–1,4–glycosidic bond between N–acetylglucosamine (NAG) and the N–acetylmuramic acid (NAM) in

the polysaccharides of certain bacterial cell wall; therefore it inhibits the growth of mainly Gram positive microorganisms, which lack an outer membrane (Salton and Pavlik, 1960). Gram negative bacteria are less susceptible to lysozyme because the outer membrane prevents the access of lysozyme to the target site (Salton and Pavlik, 1960). Among Gram negative bacteria, some genera such as *Salmonella* and *Shigella* are the most sensitive to lysozyme (Peterson and Hartsell, 1955). Ko *et al.* (2008) showed that combining lysozyme with compounds such as ethylenediaminetetraacetic acid (EDTA) and ovotransferrin can be affective against pathogenic organisms, such as *E. coli* O157:H7 and *L. monocytogenes*.

Lysozyme is stable at a wide pH range and heating temperatures; therefore it has a great potential to serve as a natural food preservative (Mine, 1995). For example, it has been shown to be effective at preventing the growth of *Bacillus cereus* and the fermentation of *Clostridium tyrobutyricum* during cheese production (Hughey and Johnson, 1987). Sung *et al.* (2011) demonstrated that adding 2 mg/ml lysozyme to milk and ground beef suppressed the growth of *B. anthracis* 3.3 and 6.5–fold, respectively. Lysozyme carbohydrate hydrolysis activity can also be exploited for lysing starter bacteria during cheese ripening (Klein and Lortal, 1999), and it is known to associate with caseins by electrostatic interactions (De Roos *et al.*, 1998) being thereby transferred to the curd.

In Europe, lysozyme has been classified as a food additive that may be used and labeled as a food ingredient. The use of lysozyme in food packaging has been studied intensively over the last decade, as reviewed in Fernandez *et al.* (2008). For example, Park *et al.* (2004) reported on the effective incorporation of lysozyme into chitosan films. Lysozyme was also incorporated into sodium alginate (Cha *et al.*, 2002), fish–skin gelatin (Bower *et al.*, 2006), and whey protein isolate (Min *et al.*, 2005), demonstrating antimicrobial activity. Recently, De Souza *et al.* (2010) developed films made of sodium caseinate with a controlled release of lysozyme. Zhong *et al.* (2009) developed a sustained release model of lysozyme from zein microcapsules produced by a supercritical anti–solvent process. In conclusion, lysozyme is a promising, natural way to provide antimicrobial effects during food storage and to enhance food safety.

There is evidence suggesting lysozyme antimicrobial activity is not only enzymatic, but can be caused by membrane disruption from lysozyme peptides. Pelligrini *et al.* (1997) showed that lysozyme contains peptide sequences which can induce non–catalytic bacterial death. Clostripain–digested lysozyme yielded a 15 amino acid peptide (amino acids 98−112) with antimicrobial activity without muramidase activity. During *et al.* (1999) also identified amphipathic peptide stretches that displayed antimicrobial activity without any enzymatic activity. Peptide A4 (amino acids 143−155) displayed a strong bactericidal and fungistatic activity, whereas peptide A23 (amino acids 126−141) was only active against fungi.

2.3.2.2 Ovomucoid

Ovomucoid has a molecular weight of 20.1 kDa and pI of 4.82. This glycoprotein contains both sialyloligosaccharides and sulphated oligosaccharides (Mine, 1995), and nine disulfide bonds. Ovomucoid is defined as a protease inhibitor. Protease inhibitors are classified by families based on amino acid similarities, and families are grouped together in clans. Each protease family has a different protease–inhibition mechanism. In egg white, protease inhibitors are considered as part of embryogenesis (Colella *et al.* 1989; Saxena and Tayyab, 1997). Chicken ovomucoid was capable of attachment to viruses such as hen Newcastle disease virus (Tsuge *et al.,* 1996) and to viruses from aquatic birds (Matrosovich *et al.,* 1999). However, the mechanisms of those egg white proteases inhibitors as antimicrobial have not been understood and the sites of action have not been identified. Ovomucoid belongs to the family of inhibitors that hold one or numerous Kazal–like units. Kazal like units are defined as a consensus sequence of six conserved cysteines which take part in disulfide bonds. Kazal–like inhibitors have a surface peptide bond called the "reactive site" which specifically interacts with the active site of the cognate protease (Laskowski and Kato, 1980). As a result of this interface, the hydrolysis of the peptide bond and the formation of an enzyme–inhibitor complex occur. The reaction is reversible and the dissociation allows for the release of active protease and

cleaved inactive inhibitor. Ovomucoid possess three Kazal domains and has been shown to inhibit trypsin and chymotrypsin (Feeney *et al.,* 1963; Kato *et al.,* 1987).

Ovomucoid purification was initially achieved by trichloracetic acid and acetone precipitation as a primary step (Lineweaver and Murray, 1947). Ovomucoid can be further purified by numerous cold acetone purification steps (Lineweaver and Murray, 1947; Kato *et al.,* 1987) and chromatographed onto a PD–10 size exclusion column (Bogard *et al.,* 1980) followed by a carboxymethyl–Sepharose chromatography (Kato *et al.,* 1987). Ovomucoid from fresh egg whites was shown to be chromatographically purified using a three–step method involving carboxymethyl (CM)–cellulose/ DEAE–cellulose/CM–cellulose (Rhodes *et al.,* 1958).

2.3.2.3 Ovotransferrin

2.3.2.3.1 Physical and chemical properties

Ovotransferrin comprises 12% of the total egg white proteins, consists of 686 amino acids with 15 disulfide bridges, has a molecular weight of 76–80 kDa and an isoelectric point of 6.1 (Williams *et al.*, 1982; Aisen, 1980). First characterized by Schade and Caroline (1944), ovotransferrin was initially named conalbumin. It was renamed ovotransferrin when it was identified as a member of the iron binding transferrin family (Williams, 1968).

Ovotransferrin is synthesized in the oviduct, under steroid control of the transferrin gene. The transferrin gene also controls the syntheses of serum transferrin by the liver, which is present in avian blood serum (Stevens, 1991; Li-Chan *et al*., 1995). The only difference between the liver transferrin and the oviduct ovotransferrin is the glycosylation of ovotransferrin. Ovotransferrin has one N–glycan bound to Asn473 (Williams *et al.*, 1982), which represents about 3% of the mass of the native protein. The glycan of ovotransferrin is composed of four residues of mannose and four residues of N–acetylglucosamine (Spik *et al.*, 1988).

The nucleotide sequence of a cDNA copy of ovotransferrin mRNA has been determined by Jeltsch and Chambon (1982). It was observed that ovotransferrin mRNA contains 2376 nucleotides split into

17 exons, coding for 705 amino acids. The signal peptide is comprised of 19 amino acids and is cleaved from the protein precursor, leaving the mature, 686 amino acid ovotransferrin molecule (Thibodeau *et al.*, 1978). Jeltsch *et al.* (1987) determined the DNA sequence of ovotransferrin, containing 10567 nucleotide base pairs.

The structure of both iron free (apo) and iron bound (holo) ovotransferrin has been determined by X–ray crystallography as can be seen on Fig. 1 (Mizutani *et al.*, 2011). The polypeptide chain is comprised of two homologous folded lobes: the N–terminal lobe (residues 1–332) and C–terminal lobe (residues 342–686), each containing a single iron binding site. The two lobes are linked by an α–helix of nine amino acid residues (residues 333–341) (Kurokawa *et al.*, 1995).

Each lobe consists of two unique, similar–sized α/β domains (N–terminal lobe, N1 and N2 domains; C–terminal lobe, C1 and C2 domains). The two domains are linked by two anti–parallel β–strands that allow them to open and close by a hinge (Kurokawa *et al.*, 1995). The N and C lobes share 37.4% sequence homology and the main differences between the two lobes are in the loop regions, which differ by sequence insertions and deletions in the primary structure (Williams *et al.*, 1982; Jeltsch *et al.*, 1987).
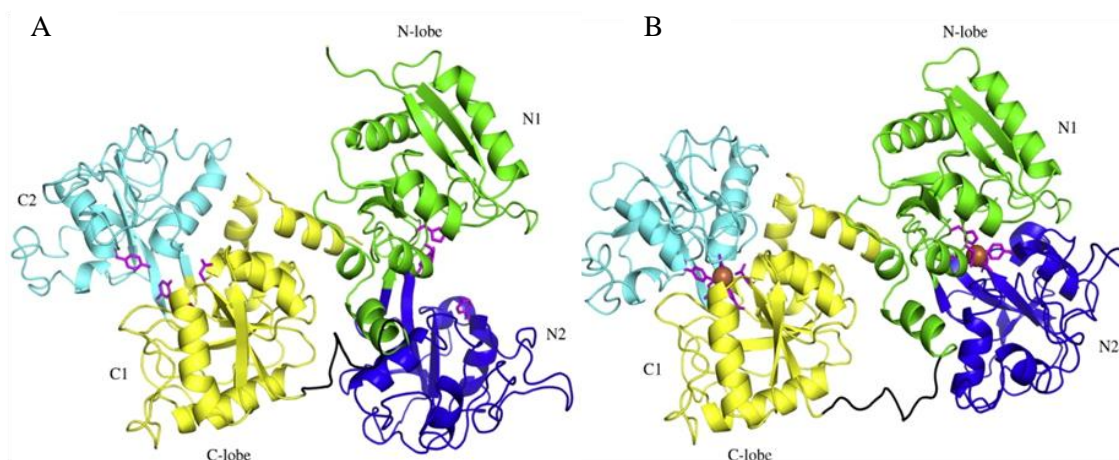
Figure 1 The change in structure of chicken ovotransferrins upon iron binding A) Apo–ovotransferrin B) Holo–ovotransferrin. Each domain is shown in different color: Domains N1 (residues 1–91 and 247–332), C1 (343–429 and 589–686), N2 (92–246) and C2 (430–588) are shown in green, blue, yellow and cyan, respectively. Loops connecting two lobes are shown in black. Iron–binding residues, Asp, two Tyr and His, are shown in magenta as stick models (Software: PyMOL, Schrödinger) (© Biochimica et Biophysica Acta, 2011, adapted by permission).

2.3.2.3.2 Iron binding and release

Ovotransferrin is found as colorless, iron–free form in the hen egg white. Upon iron binding, its color changes to salmon–pink (Alderton *et al.*, 1946). The binding of iron to ovotransferrin requires one molecule of CO_2 as CO_3^{-2} or HCO_3^{-1} per atom of Fe^{3+}; therefore bicarbonate is known as a synergistic anion (Schalabach and Bates, 1975). Bicarbonate is a prerequisite for iron binding since it serves as a bridging ligand between the protein and the iron (Aisen, 1980). Additionally, decarbonation is a prerequisite for iron release (El Hage Chahine and Pakadaman, 1995).

Ovotransferrin possesses two binding sites for ferric iron: one binding site is located in the C–terminal lobe and one is located in the N–terminal lobe. The iron–binding site is located in the interdomain cleft in each lobe. The ligands for the bound Fe^{3+} ion are the same in both lobes, comprising two tyrosine residues, one aspartic acid and one histidine (Tyr92, Tyr191, Asp60 and His250 in the N–lobe), together with two oxygens from the synergistic bicarbonate, as can be seen on Fig 2.

Figure 2 A diagram of the Fe^{3+} and bicarbonate binding site of the N–terminal lobe of ovotransferrin. Hydrogen bonds are shown by broken lines and their lengths are indicated in A° (© Journal of Molecular Biology, 1995, adapted by permission).
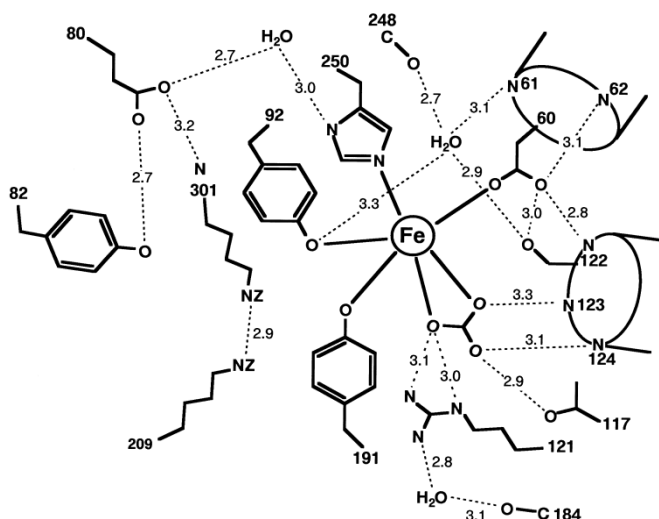
Though both lobes have the capability to reversibly bind Fe^{3+}, they have different affinities for iron; the iron–binding constant is 1.5×10^{18} M^{-1} for the C–terminal lobe and 1.5×10^{14} M^{-1} for the N–terminal lobe (Lin *et al.*, 1994).

Upon iron–binding and release, each ovotransferrin lobe undergoes a significant conformational change. Both interdomain clefts are open before iron binding and close when iron is incorporated into the iron–binding site (Anderson *et al.*, 1990). The major conformational changes occur in the hinge regions on the two β–strands that link the domains of each lobe. In addition to the domain opening in the two lobes, there is a 7° rotation of the C1–domain relative to the N1–domain (Kurokawa *et al.*, 1999).

Ovotransferrin iron binding and release is significantly affected by pH. Iron binding capacity of ovotransferrin increased as the pH of the solution increased from 6.5 to 9.5 (Abdallah and EL Hage Chahine, 1998). Iron release from ovotransferrin occurs under the acidic pH range (pH 3.0 to 6.0). The pH has different effects on C–terminal and N–terminal lobes. At alkaline pH, iron preferentially occupies the N–terminal binding site. At acidic pH, the N–lobe of ovotransferrin show weaker binding stability and faster Fe^{3+} release than the C–lobe (Griffiths and Humphreys, 1977; Mizutani *et al.*, 2011).

Holo–ovotransferrin is relatively stable to proteolytic hydrolysis, and chemical and thermal denaturation. Iron affinity of apo–ovotransferrin is decreased under denaturing conditions because of the change in the structure of native–ovotransferrin, which is required for the formation of holo–ovotransferrin (Fraenkel–Conrat, 1950).

Understanding the chemical and physical mechanism of iron uptake and release of ovotransferrin is crucial for understanding its antimicrobial activity, as elaborated in the next section.

2.3.2.3.3 Antimicrobial activity

Chicken ovotransferrin has antimicrobial activity against different Gram negative and Gram positive microorganisms including *Escherichia coli* (Schade and Caroline, 1944), *Pseudomonas* spp.,

Streptococcus mutans (Valenti *et al.*, 1983), *Staphylococcus aureus, Bacillus cereus* (Ibrahim *et al.*,1997) and *Salmonella enteritidis* (Baron *et al.*, 2000).

The mechanism by which ovotransferrin exerts its antimicrobial activity is controversial.

The first mechanism suggested for its antimicrobial activity was the iron–binding capability, which limits the availability of iron required for microbial growth (Alderton *et al.*, 1946; Fraenkel–Conrat, 1950). This function was shown to be depended on iron concentration in the environment, on the degree of ovotransferrin iron saturation, presence of synergistic ions, pH, and on the synthesis of bacterial siderophores, which compete with ovotransferrin for iron acquisition (Valenti *et al.*, 1983). Iron is essential for bacterial growth, since it has a critical role in electron transport, metabolism, DNA metabolism, and regulation of gene expression (Crosa *et al.*, 2004).

There are several studies that contradict the iron–binding capacity as the ovotransferrin antimicrobial mechanism. According to this mechanism, antimicrobial activity should be eliminated once ovotransferrin is metal saturated. However, ovotransferrin saturated with metals as such zinc and iron was reported to retain antimicrobial activity (Ibrahim, 1997; Valenti *et al.*, 1983; 1987). For example, Zn^{2+} saturated ovotransferrin was found to be more bactericidal than apo–ovotransferrin and other metal saturated ovotransferrin (Valenti *et al.*, 1987). This data suggested that a second antimicrobial mechanism of ovotransferrin exists.

The second antimicrobial mechanism of ovotransferrin is ascribed to direct interactions of ovotransferrin with the bacterial surface. OTAP–92, a short cationic sequence located in the N–terminal domain of ovotransferrin, was found to damage the outer membranes of microorganisms (Ibrahim *et al.*, 1998; Ibrahim *et al.*, 2000). The cationic antimicrobial peptide can cross the outer membrane and damage the trans–membrane electrochemical potential; therefore it exhibits bactericidal properties against Gram–negative bacteria (Ibrahim *et al.*, 2000). In order for the peptide to be antimicrobial, the preservation of its tertiary structure is essential. When disulfide bonds of OTAP–92 were reduced, the bactericidal property was lost (Ibrahim *et al.*, 1998).

In conclusion, further studies to clarify the mechanism(s) of antibacterial activity of ovotransferrin are needed.

2.3.2.3.3.1 Ovotransferrin antimicrobial activity against *S. aureus* and *E. coli*:

The use of ovotransferrin as an antimicrobial agent against *E. coli* and *S. aureus* has already been evaluated with conflicting results. Ko *et al.* (2008) found that though apo–ovotransferrin had bacteriostatic activity against *E. coli* O157:H7 in brain heart infusion (BHI), no activity was observed on *E. coli* O157:H7 when it was applied to the surface of commercial hams and pork chops. Recently, the antimicrobial activity of κ–carrageenan–based film (κCF) containing native ovotransferrin against "laboratory strains", *E. coli* K–12 and *S. aureus* ATCC 12692 was investigated. The κCF–ovotransferrin showed a slight significant antimicrobial activity against *E. coli* in the presence of 5 mM EDTA. However, there were only weak inhibitory effects against *S. aureus* (Seol *et al.*, 2009).

Valenti *et al.*, (1980) reported that 50 strains of *S. aureus* were resistant to native ovotransferrin due to efficient iron transport systems, as determined by comparing the bacterial growth of a media containing ovotransferrin with a media where iron was precipitated with chromium salts. Similar growth rate was observed for both medias, suggesting iron binding as a mode of antimicrobial action of ovotransferrin. An alternative mechanism was suggested by Ibrahim *et al.* (1997 and 2000), who showed that the N–terminal lobe of ovotransferrin had bactericidal activity against *S. aureus* IFO 14462, regardless of iron saturation. The antimicrobial mechanism that was suggested was damage to the bacterial outer membrane from direct interactions of OTAP–92, a short cationic sequence located in the N–terminal domain of ovotransferrin.

There have been a few studies regarding human transferrin and iron binding as the antimicrobial activity mechanism against *S. aureus*. Human transferrin has higher affinity for iron than ovotransferrin, with an association constant to iron of 10^{32} M^{-1}, more than twice as high as ovotransferrin (Lin *et al.*, 1994). Lindsay *et al.* (1995) showed that *S. aureus* ATCC 12600 can extract iron bound to human transferrin by labeling transferrin with ^{55}Fe. They observed that

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growing cultures of *S. aureus* could slowly take up radioactive iron during mid– to late–exponential phase of growth. This process did not require direct contact between the cell and the labeled transferrin. Interestingly, by examining the exponential growth–phase supernatant, detectable levels of reducing ability or protease activity were not observed, suggesting that the component of the supernatant responsible for the uptake of Fe bound to transferrin was a siderophore, a high–affinity extracellular ferric chelator. The iron uptake mechanism of *S. aureus* was investigated by Kadurugamuwa *et al.* (1987), who studied the growth kinetics and the disappearance of iron from the culture supernatant of *S. aureus* ATCC 12600. It was observed that *S. aureus* grew under iron–depleted conditions, suggesting that the iron requirement of *S. aureus* is relatively low. Another possibility is that *S. aureus* can alter its metabolic pathways and reduce the envelope–associated iron so that less iron is required by the organism.

Overall, it seems that more research has to be done in order to determine the iron requirements of *S. aureus,* as a first step towards understanding whether the antimicrobial mechanism of ovotransferrin is related to its iron binding capacity.

The antimicrobial effect of ovotransferrin against *E. coli* has been studied more extensively, both on laboratory strains and clinical isolated pathogenic strains, including O157:H7.

For example, the effect of citrate as a chelating ion on the antimicrobial activity of ovotransferrin was studied with *E. coli* K–12. Since *E. coli* has an iron transport system mediated by citrate (Frost and Rosemberg, 1973), it can compete with the antibacterial activity of ovotransferrin.

Iron deprivation as the mechanism for the antimicrobial activity of ovotransferrin against *E. coli* has been controversial as well. Ibrahim *et al.* (1998 and 2000), suggested that the N–terminal lobe of ovotransferrin had bactericidal activity against *E. coli* K–12, regardless of iron saturation. However, Ko *et al.* (2008) demonstrated that apo–ovotransferrin (combined with $NaHCO_3$ and EDTA) had the potential to inhibit *E. coli* O157:H7 growth and that holo–ovotransferrin had little or no inhibitory activity against this strain.

Gram negative bacteria studied under conditions of iron deprivation have been shown to increase expression of iron–regulated outer membrane proteins (Neilands, 1982). It has been observed that the iron metabolism of all *E. coli* strains utilizes siderophores as one of the main mechanisms to solubilize iron prior to transport (Earhart, 1996). When *E. coli* is present in an environment that is iron deficient, it secretes a catechol–type siderophore, enterobactin (also known as enterochelin), which serves to mobilize this essential element (O'Brien and Gibson, 1970).

In order to further understand the antimicrobial activity of ovotransferrin against *E. coli*, more research, in terms of iron uptake characteristics in the presence of ovotransferrin, has to be executed before applying ovotransferrin to food matrices.

2.3.2.3.4 Purification and identification techniques

Several methods for separation of ovotransferrin from egg white have been suggested by different researchers. In the past, ovotransferrin was separated from egg white by isolating it with aqueous and ethanol fractions (Bain and Deutsch, 1948; Warner and Weber, 1953), by precipitation with ammonium sulfate, or by coagulating ovalbumin (Azari and Baugh, 1967). Nevertheless, these methods had some disadvantages such as the need for acidic conditions to obtain the ammonium sulfate precipitation, while the ethanol precipitation denatured ovotransferrin, and the purity of the resulting products was relatively low (Vachier *et al.*, 1995). In order to overcome these drawbacks, chromatography methods was developed. Liquid chromatography has a significant part in the fractionation of ovotransferrin either by ion exchange chromatography or by affinity chromatography (Croguennec *et al.*, 2001). Ion exchange chromatography has been the predominate method for protein purification due to its simplicity and scale–up possibilities (Larive *et al.*, 1999). Except for lysozyme and avidin, ovotransferrin has the highest isoelectric point of all egg white proteins, therefore its extraction by ion exchange chromatography had been carried out mostly on cation exchangers. Fractionated ovotransferrin from egg white on a CM cellulose column resulted in ovotransferrin contaminated by globulins (Rhodes *et al.*, 1958). By adding further precipitation and crystallization steps after ovotransferrin elution, a purer protein was obtained (Azari and Baugh,

1967). Cation exchange chromatography had been also used for ovotransferrin purification, especially for industrial scale production of ovotransferrin from egg white devoid of lysozyme. The drawback of this method was that due to the small difference of pH between the ovotransferrin pI and the pH used for ovotransferrin fixation to the resin, the equilibration time was more than 7 hours (Guerin and Brule, 1992).

Ovotransferrin from undiluted, blended egg white was separated by a single step chromatographic method using an immobilized metal affinity chromatography (Al–Mashikhi and Nakai, 1987). In general, this method is a common and efficient method for the isolation and purification of proteins, due to its high discriminating potential (Porath *et al.*, 1975).

Chung *et al.* (1991) used an affinity method with bifunctional dye–ligand chromatography using DEAE Affi–Gel Blue as initial step to fractionate ovotransferrin. The drawback of this method was that it was only practical for small–scale purification and therefore only small quantities were isolated. In addition, affinity methods are more expensive than ion exchangers. Guerin and Brule (1992) developed a pilot scale method to purify ovotransferrin using a Duolite C–476 resin, but the process was time consuming.

Ovotransferrin was separated by a 2–step chromatographic procedure involving gel permeation on a Superose–6 Prep grade column and anion–exchange chromatography on a Q–Superose Fast Flow. The purification yield was over 60%, with 80% purity. Further electrospray ionization mass spectrum showed different ovotransferrin species (Awadé *et al.*, 1994). Another 2–step chromatographic procedure for the preparation of hen egg white ovotransferrin using an anion exchanger consists of a preliminary step of displacement chromatography on a Q Sepharose Fast Flow column was described by Croguennec *et al.*, (2001). The pre–purification of ovotransferrin and further purification by a second conventional preparative chromatography performed on the same Q Sepharose FF column resulted in homogeneous ovotransferrin in electrophoresis and analytical chromatography. Its purity was 97.5% and its amino acid composition was close to that obtained from the nucleotide sequence of ovotransferrin. In a recent study, an 80% pure ovotransferrin was isolated from ovomucin–free egg

white by anion–exchange column followed by cation–exchange chromatography (Omana *et al.*, 2010). It is important to stress that those procedures using ion–exchange chromatography or affinity chromatography are only suitable for laboratory scale. The application of these methods on a pilot– scale is difficult, because these methods are tedious and not cost efficient (Awadé, 1996).

Ko and Ahn (2008) proposed a large–scale purification method in which egg white was treated in order to convert ovotransferrin to holo–form by addition of iron chloride. The holo–ovotransferrin was separated from other proteins by precipitation with ethanol and then converted into apo– ovotransferrin using an ion exchange resin; the resulting apo–ovotransferrin was 80% pure as determined by SDS–PAGE.

2.3.2.4 Ovalbumin

Ovalbumin is the most abundant protein in egg white, comprises 54% of the total egg white protein weight. It is a 45 kDa phospho–glycoprotein comprised of 385 amino acids residues, that fold into a globular conformation with a high secondary structure content (30.6% α–helix and 31.4% β–strand) (Stein *et al.*, 1991). Though ovalbumin is classified as a serpin (serine proteinase inhibitor) according to sequence homology classification, protease inhibitor activity is absent in its native form (Hunt and Dayhoff, 1980; Wright, 1984; Stein *et al.*, 1990). Despite that, Mellet *et al.* (1996) showed that heating ovalbumin for 30 min at 97 °C altered it into a protease inhibitor. This transformed ovalbumin resembles serpins by its function as a reversible competitive proteinase inhibitor, though it differs from typical serpins by its inability to form irreversible complexes. Besides its protease inhibitory function, ovalbumin–derived peptides have been studied for their antimicrobial activity. Peptides produced by the enzymatic digestion of ovalbumin were strongly active against *Bacillus subtilis* and to a lesser extent against *E. coli, Bordetella bronchiseptica, Pseudomonas aeruginosa, Serratia marcescens*, and *Candida albicans* (Pellegrini *et al.*, 2004).

Ovalbumin has immune–modulating activity, inducing the release of tumor necrosis factor in a dose dependent manner *in vitro*, when modified with dicarbonyl methylglyoxyl (Fan *et al.*, 2003), and

immunogenic ovalbumin peptides have been used to increase immune responses for cancer immunotherapy (He *et al.*, 2003). Additionally, it has been reported that certain ovalbumin–derived peptides can contribute to the control of hypertension by exerting vaso–relaxing effects (Davalos *et al.*, 2004). Two antihypertensive peptides, ovokinin (OA 358−365) and ovokinin (OA 2−7), were isolated by peptic digestion and by chymotrypsin digestion, respectively (Fujita *et al.*, 1995; Matoba *et al.*, 1999).

Ovalbumin has been a concern in the food industry, since it was classified as a major food allergen in the 1980's. Another concern with ovalbumin involves its structure alternation into S–ovalbumin during storage of eggs. S–ovalbumin is more stable and compact (Mine, 1995), and results in a heat–induced gel with poor strength that does not coagulate as effectively upon processing (Yamasaki *et al.*, 2003).

2.3.2.5 Sialic acid

Sialic acid is a nine–carbon carboxylated sugar molecule that serves as part of the structure of carbohydrates including glycoproteins, glycolipids, and keratan sulfate proteoglycan (Corfield and Schauer 1982; Stuhlsatz *et al.,* 1989). Many types of sialic acid are present in all vertebrate species. The most common sialic acid is N–acetylneuraminic acid, which is believed to be the biosynthetic precursor of all other types of sialic acid (Varki, 1992).

Sialic acid has a broad physiological functionality. It contributes to cell adhesiveness, enzyme inhibition, hormonal action, antigenicity, and synaptic transmission (Reutter *et al.,* 1982).

Sialic acid was purified from egg fractions, including chalaza and yolk membrane (Itoh *et al.,* 1989; Juneja *et al.*, 1991). Sialic acid was found to serve as a key component of pharmaceutical and food products, therefore several purification techniques have been developed (Itoh *et al.*, 1989; Juneja *et al.*, l99l; Koketsu *et al.*, 1993). Sialic acid contributes to cell adhesiveness and enzyme inhibition; therefore can serve as a natural antimicrobial agent (Reutter *et al.*, 1982).

2.4 *Escherichia coli* O157:H7

Enterohemorrhagic (EHEC) *Escherichia coli* (*E. coli*) O157:H7 is one of the five most common foodborne pathogens. It was first identified as a human pathogen causing outbreaks of bloody diarrhea in the USA, in 1982 (Wells *et al.*, 1983; Riley *et al.*, 1983). The USA Center for Disease Control and Prevention has estimated that *E. coli* O157:H7 infections cause approximately 73,000 illnesses and 60 deaths yearly in the United States (Mead *et al.*, 1999), costing the health care system 405 million dollars each year (Frenzen *et al.*, 2005).

E. coli O157:H7 human infections have vast clinical outcomes, ranging from asymptomatic cases to death. The majority of cases are self–resolved non–bloody diarrhea. However, in some events bloody diarrhea or hemorrhagic colitis may appear in 1–3 days, with 5% chances to progress to a life–threatening issue (Banatvala *et al.*, 2001). Several approaches for therapy have been studied including the use of antibiotics and vaccination. However, there is no specific treatment for *E. coli* O157:H7 infection and the use of antibiotics may not always be effective. The focus has been on treatment of symptoms and preventing systemic complications; therefore, the best strategy is prevention and control of *E. coli* O157:H7 reservoirs and infections pathways.

Bovine food products and fresh produce contaminated with bovine waste are the most common food sources for disease outbreaks (Dunn *et al.*, 2004; Cho *et al.*, 2006 and Hancock *et al.*, 1997).This contamination may develop from feces and arise from the presence of animals on fields, irrigation, staff, or during transporting, processing and packaging. Transmission of *E. coli* O157:H7 between cattle is mainly through the fecal–oral route, although transmission may occur indirectly through an environmental reservoir (Chase *et al.*, 2008). *E. coli* O157:H7 naturally colonizes the gastrointestinal tracts of healthy cattle. The site of colonization is the lymphoid follicle–dense mucosa at the terminal rectum, called the rectoanal junction mucosa (Lim *et al.*, 2007 and Naylor *et al.*, 2003). This data emphasize that elimination of *E. coli* O157:H7 outbreaks lies within prevention and control in cattle. To control *E. coli* O157:H7 on the farm, improvement of cattle management practices, the identification of inhibitory feeds, immunization, the utilization of feeding additives, and the use of

probiotic cultures have been proposed (Chase *et al.*, 2008). Despite of those interventions, recalls and outbreaks are still increasing.

The ability to colonize the bovine gastrointestinal tract and survive in the acidic environment of the stomach requires that *E. coli* O157:H7 adapt to a wide variety of conditions. The adaptation to acidic environment of the stomach increases the chance of bacteria to colonize the intestines and cause infection. Acid resistance is associated with lowering the infectious dose of enteric pathogens (Schlech *et al.*, 1993).The infectious dose of *E. coli* O157:H7 is between 10 to 100 cells (Reilly, 1998).

Three major virulence factors of *E. coli* O157:H7 have been identified including Shiga toxins, products of the pathogenicity island called the locus of enterocyte effacement, and products of the F–like plasmid pO157.

E. coli O157:H7 may have descended from the non–toxigenic and less virulent strain *E. coli* O55:H7 (Wick *et al.*, 2005). *E. coli* O157:H7 has developed through four consecutive events; (i) acquisition of an *stx2*–containing bacteriophage, (ii) acquisition of pO157 and the *rfb* region, (iii) acquisition of the *stx1*–containing bacteriophage, and (iv) loss of the ability to ferment D–sorbitol and loss of beta–glucuronidase activity (Reviewed in Lim *et al.*, 2009).

A variety of molecular subtyping methods have been developed to improve the understanding of the epidemiology of *E. coli* O157:H7 outbreaks. Among them, pulsed field gel electrophoresis (PFGE) method was standardized by CDC and has been applied successfully to discriminate outbreak associated or unrelated infections since 1993 (Barrett *et al.*, 1994).

2.5 *Staphylococcus aureus* COL

Staphylococcus aureus COL is a pathogenic, methicillin resistant *S. aureus* (MRSA) strain. It is a facultative anaerobic Gram–positive coccus, that is non–motile, and catalase and coagulase positive.

Methicillin resistant *S. aureus* was discovered in the United Kingdom in 1961, and by the mid–1970s had become endemic in many countries (Voss and Doebbeling, 1995). More than 60% of all *S.*

aureus isolates are resistant to methicillin and some strains have developed resistance to more than 20 different antimicrobial agents (Lowy, 1998). These strains produce numerous toxins including super antigens that cause unique disease entities such as toxic–shock syndrome and staphylococcal scarlet fever (Gill *et al.*, 2005). Some strains of MRSA have been recognized as epidemic strains, associating with a higher prevalence and spreading within hospitals, between hospitals, and between countries (Mulligan *et al.*, 1993; Aires de Sousa *et al.*, 1998). Transmission of MRSA is thought to occur primarily from colonized or infected persons to other persons, whereas transmission through food products has not been studied extensively. Despite that, there are publications on the epidemiological aspects of MRSA infections in animals, for example, pet dogs (Cefai *et al.*, 1994) and MRSA infections in dairy herds have been reported (Devriese and Hommez, 1975). More recently, Lee (2003) reported on MRSA present in dairy cows and chickens. The presence of MRSA in those animals can lead to an increasing prevalence of MRSA in food, and therefore it should be further studied as a food–borne pathogen.

In general, pathogenic *S. aureus* strains are invasive, able to produce staphylococcal enterotoxins and resistant to antibiotics; therefore *S. aureus* foodborne illness has been an important issue in the food safety community. *S. aureus* contamination can be avoided by heat treatment of food. Despite that, it remains a major source of foodborne illness because of its ability to contaminate food products during preparation, processing and packaging. Most importantly, even after *S. aureus* are killed, its toxins remain in the food matrix, causing foodborne disease. The symptoms of staphylococcal foodborne illness are abdominal cramps, nausea, vomiting, sometimes followed by diarrhea. The onset of symptoms is rapid (from 30 min to 8 h) and usually spontaneous remission is observed after 24 hr (reviewed in Le Loir *et al.*, 2003).

S. aureus virulence involves the temporally coordinated synthesis of a large number of surface and secreted proteins. *S. aureus* is able to grow in a wide range of temperatures (7 to 48.5°C with an optimum of 30 to 37°C (Schmitt *et al.*, 1990), and sodium chloride concentrations (up to 15% NaCl (Sutherland *et al.*, 1994)).

In all cases of *S. aureus* foodborne illness, the food was exposed to temperatures that allowed *S. aureus* to grow. Many different foods such as milk and cream, cream–filled pastries, butter, ham, cheeses, sausages, canned meat, salads, cooked meals and sandwich fillings can be a good growth medium for *S. aureus* (Wieneke *et al.*, 1993; Genigeorgis, 1989).

2.6 Egg white proteomics

The proteome of a cell or an organelle provides data about the protein array expressed under specific physiological and chemical conditions during a certain time (Wasinger *et al.*, 1995).

Two–dimensional gel electrophoresis (2DE) combined with mass spectrometry, as a high–resolution technique for proteome analysis, has been successfully applied in egg white protein characterization (Wysocki *et al.*, 2005). In 2DE proteins are being separated and visualized according to their isoelectric point and mass. This technique can separate post–translational modifications of intact proteins, including phosphorylation or glycosylation. Mass spectrometers measure the mass/charge ratio of the analytes (reviewed in Wysocki *et al.*, 2005). The complex peptide mixtures are separated by nano–scale reversed phase liquid chromatography and ionized by applying a strong electric potential at the outlet of the capillary column, a process called electrospray ionization (ESI). The protonated ions generated by the electrospray process enter the evacuated mass spectrometer through a small hole in the source interface. The most widely used ion activation and dissociation processes is gas–phase collision–activated dissociation. Additional dissociation techniques are infrared multiphoton dissociation (IRMPD) and electron capture dissociation (ECD). The next step is to obtain sequence information from the most intense peptide ions. The peptide ions are isolated in order, resonantly excited and collided with helium, which causes them to fragment at preferred sites along the backbone, with detection by MS/MS (Mann, 2007). The application of mass spectrometry to egg white proteomics takes advantage of the vast and growing array of genome and protein data stored in databases. To identify peptides, database searching programs compare each MS/MS spectrum against theoretical spectra of candidate peptide sequences represented in a protein database,

and a score is assigned to rank the most likely peptide assignments.

Desert *et al.* (2001) were first to conduct a proteome–level investigation of hen egg white which led to the characterization of small acidic proteins, among them an 18 kDa protein known as Ch21. Guerin–Dubiard *et al.* (2006) identified 16 proteins; two of them had never been previously detected in hen egg white, such as Tenp, a protein with strong homology with a bacterial permeability–increasing protein family (BPI), and VMO–1, an outer layer vitelline membrane protein. Additionally they showed that 13 proteins had a very wide polymorphism: ovotransferrin was found in five isoforms. Subsequently, Raikos *et al.* (2007) investigated the whole egg proteins using 2D gel electrophoresis combined with MALDI–TOF mass spectrometry to identify selected protein spots using peptide mass fingerprinting. Mann (2007) detected 78 egg white proteins using 1–DE and LC–MS/MS, and 54 proteins were identified for the first time. Proteins which were previously only characterized by partial sequences, such as b–ovomucin or ovalbumin X, were identified and confirmed with a high coverage by MS–sequenced peptides.

Since ovalbumin, ovotransferrin and ovomucoid make up approximately 75% of the total egg white protein, it is difficult to detect low abundance proteins by mass spectrometry, because the peptides of these few proteins tend to mask other signals (Mann and Mann, 2011). D'Ambrosio *et al.* (2008) resolved this issue by using 2–DE combined with a protein enrichment technology, using peptide ligand libraries. They reported a total of 148 proteins from egg white. The use of this technique allowed the discovery and identification of a large number of previously unreported egg white proteins. However, according to Mann and Mann (2011) this technique was reported to be only suitable for soluble proteins. Additionally, the composition of the proteome can be modified in an unknown and unpredictable way, which makes it impossible to determine the absolute quantity of the proteins (Mann and Mann, 2011). In the most recent study regarding egg white proteomics, Mann and Mann (2011) used a novel dual pressure linear ion trap instrument, the LTQ Orbitrap Velos to identify 158 egg white proteins. They reported that this instrument had increased sensitivity and was fast enough to isolate and fragment ten or more peaks simultaneously with the acquisition of one high

resolution mass full scan spectrum.

In conclusion, high–throughput mass spectrometry based proteomics has significantly increased our knowledge of egg white protein composition. Mass spectrometry is an advanced technique to identify proteins with unique biological functions that were not determined before by more conventional analysis such as electrophoresis or chromatography; therefore mass spectrometry can serve as an outstanding tool for food science and technology research.

3. MATERIALS AND METHODS

Eggs: Four to six day old Chilean tinamou eggs were purchased from a local farmer; a few day old emu eggs were obtained from UBC farm and regular medium size chicken eggs were purchased from the local supermarket. All egg whites were separated from yolks and stored at –20°C. Egg whites samples were defrosted prior to each assay and homogenized for 30 min with a medium velocity magnetic stirring plate.

3.1 Egg white characterization

3.1.1 Determination of protein content in egg white

3.1.1.1 Bradford protein assay

Protein content of tinamou and chicken egg whites and tinamou egg white FPLC fractions was determined by Bio–Rad protein assay (Bradford, 1976). Three chicken egg whites and three tinamou egg whites were homogenized and samples of 0.1 –0.5 mg/ml were made in triplicates.

The concentrate Bio–Rad dye reagent, Coomassie brilliant blue G–250 dye, was diluted with deionized water in 1:4 dilution ratios and then filtered with Whatman #1 filter. An aliquot of 10 µl of each standard and sample solution and 200 µl of diluted dye reagent was pipette into separate microtiter plate wells (in triplicates). A standard curve was prepared from triplicates of bovine serum albumin (BSA, Sigma–Aldrich) at concentrations of 0.05–0.5 mg/ml. Absorbance of all standards and samples was measured at 595 nm using a microplate reader (Bio–Rad) after 5 min.

3.1.1.2 Kjeldahl method

Protein content of tinamou, chicken and emu egg whites was determined by the Kjeldahl method, according to the AOAC official method 932.08, Nitrogen in liquid eggs, N=6.25. Three homogenized egg white samples (1 gram) from chicken and tinamou were tested in triplicates, (18 samples in total). Two emu eggs were used due to their limited availability.

3.1.2 Determination of sialic acid content in egg white by the Warren method

Sialic acid content of tinamou, chicken and emu egg whites was determined by a modified Warren method (Warren, 1959), a colorimetric method based on the formation of a chromophore. Duplicate aliquots of 0.2 ml from each of two homogenized egg white were made (four replicates in total per species).

The periodate oxidation product was mixed with thiobarbituric acid, following extraction into 1–propanol instead of cyclohexanone. A standard curve was prepared from triplicates of N–acetylneuraminic acid (Sigma–Aldrich) at concentrations of 0–5 µmol /ml. Distilled deionized water was used for the blanks. Absorbance of all standards and samples was detected at 549 nm with a spectrophotometer (Shimazdu UV160–U).

3.1.3 Determination of iron content in dry ashed egg white by the ferrozine assay

Dry Ashing: Triplicate aliquots of 2 g from each of homogenized egg white were placed in pre–ashed porcelain crucibles, and heated overnight at 550° C in a muffle furnace (Thermolyne 6270). Ash was calculated as % ash /wet weight egg white.

Ferrozine assay: Iron content of pre–ashed tinamou, chicken and emu egg whites samples was determined by the ferrozine assay, adapted from Lysionek *et al.* (1998) with a few modifications.

Ash was dissolved with 2 ml of 2N HCl and distilled water was added to a volume of 10 ml. One ml of this dilution was combined with 1 ml of each 0.2 N ammonium hydroxide, 2 % mercaptoacetic acid, 5 M acetate–acetic acid buffer at pH 4.5 (i.e. 125.9 g NaOAc·3 H_2O + 100 ml glacial acetic acid, dilute to 500 ml with distilled water) and 0.2% Ferrozine iron reagent in solution (3–(2–pyridyl)–5,6–diphenyl–1,2,4–triazine–p,p′–disulfonic acid monosodium salt hydrate, Sigma–Aldrich), shaken and left at least 15 min at room temperature. A standard curve was prepared from triplicates of iron standard solution (Sigma–Aldrich) at concentrations of 0–2 µg iron/ml. Blanks were made using distilled water instead of egg white. Absorbance of all standards and samples was detected at 562 nm with the spectrophotometer (Shimazdu UV160–U).

3.1.4 Determination of lysozyme activity in egg white by the lysozyme activity assay

The enzymatic activity of lysozyme in tinamou egg white was measured by incubating tinamou egg white samples with lyophilized *Micrococcus lysodeikticus* (Sigma–Aldrich) as a substrate. Triplicates of the lysozyme fraction from FPLC as well as "fresh" (4 day old) and "old" (1 month old) tinamou egg white samples were tested and compared to 500 units/ml chicken lysozyme (Sigma–Aldrich).

Nine mg of dried *M. lysodeikticus* cells were suspended in 30 ml of 0.1 M potassium phosphate buffer (pH 7.0). A volume of 0.9 ml of the suspension was pre–incubated in the spectrometer (Unicam UV/Vis Spectrometer) for 2 min and the initial OD_{450} was recorded. The lysis of *M. lysodeikticus* cells was determined by monitoring the decrease in turbidity in OD_{450} (26°C) at 30 seconds time intervals, after adding 0.1 ml of various samples. The activity was presented as the rate of decrease in absorbance per minute of the initial velocity of reaction. One unit produced a change in OD_{450} of 0.001 per minute under the specified pH and temperature.

3.2 Isolation and identification of the major Chilean tinamou egg white proteins and comparison to the chicken egg white proteins

3.2.1 Separation of the egg white proteins by anion–exchange FPLC

Egg whites samples were prepared as specified by Awadé and Efstathiou (1999). Generally, ovomucin was separated from the entire egg whites by the following procedure: egg whites were diluted 10 fold with 0.02 M tris (hydroxymethyl) aminomethane HCl (Tris–HCl) buffer (pH 9.0) containing 10 mM β–mercaptoethanol. All samples were gently stirred overnight at 4°C and filtered through cellulose–acetate 0.22 µm filters prior to analysis by anion exchange chromatography.

Egg white proteins were separated with anion–exchange columns. Initially, in order to determine the separation profile, proteins were separated by HiTrap Q HP 5/1 column equilibrated with 0.02 M Tris–HCl (pH 9.0) using the FPLC system. Diluted egg whites in a volume of 500 µl were injected to the HiTrap Q HP 5/1 column. Elution was conducted by increasing 0.5 M NaCl concentration in the

same buffer as shown on Table 1. Proteins were detected at 280 nm and fractions were collected in 1 minute intervals.

Table 1 Parameters for the elution of egg white proteins from the anion–exchange column

Time (min)	Buffer A (% v/v) (0.02 M Tris–HCl, pH 9.0)	Buffer B (% v/v) (Buffer A+ 0.5 M NaCl)
0	100	0
2	90	10
3	75	25
7	80	20
7.5	67.5	32.5
8.5	67.5	32.5
9	60	40
12	60	40
13	0	100
14	0	100
15	100	0
17	100	0

Major protein fractions were collected and concentrated as following: each 15 ml eluted was concentrated in a 3 kDa molecular weight cut off centrifugal filter device (Ultra–15 Amicon) and centrifuged for 30 min at 3000 g. Filtration was repeated twice more, while adding distilled water to the retentate to 15 ml of solution. This process allowed both concentration of the fraction and desalting of buffer B (0.5 M NaCl). Aliquots were subjected to Bradford assay in order to determine the protein concentration in each fraction pre and post concentration. Following that, larger egg white aliquots (50 ml) were separated by HR Source 15Q 16/10, (GE Life sciences). Diluted egg whites in a volume of 50 ml were injected to the HR Source 15Q 16/10 column, using a super-loop. Elution was conducted by a modified profile of Table 1.

3.2.2 Separation and identification of egg white proteins by SDS–PAGE

Egg whites were diluted with water to several concentrations (0–5 mg protein/ml). FPLC samples were concentrated as described at 3.2.1. Aliquots of 20 µl of diluted egg white samples, concentrated

FPLC fractions were mixed with 20 µl sample buffer and boiled for 5 min; after centrifugation, a 10 µl aliquot of each sample supernatant was loaded into a well.

SDS–PAG electrophoresis was performed according to the method of Laemmli (1970) at 150 V for 40 min. The 12.5% SDS gel was stained with 0.1% Coomassie brilliant blue solution for 1 hour and destained twice using destaining solution (45% methanol, 10% acetic acid). Protein molecular mass markers (Fermentas) were used for molecular weight determination of the denatured reduced proteins using the Image Quant TL software. The molecular weight of each protein band was calculated by the relative mobility of band to that migrated of the molecular weight marker. That was defined by plotting the migration distances versus the logarithm of protein molecular mass (log Mr).

The ovotransferrin content of egg whites was calculated using the same software by measuring the band optical density relative to the optical density of the entire lane, which was proportional to the protein concentration.

3.2.3 Deglycosylation of concentrated FPLC fractions by Peptide:N–glycosidase F (PNGase-F)

PNGase–F kit was purchased from Biolabs. Nine microliter aliquot from each FPLC concentrated protein fraction was mixed with 1 µl of 10X glycoprotein denaturing buffer (5% SDS, 0.4 M dithiothreitol) and then denatured by heating at 100° C for 10 min. Two microliter of 10XG7 reaction buffer (0.5 M sodium phosphate pH 7.5), 2 µl 10% NP–40 detergent, distilled water, and 2 µl PNGase ($1.8*10^6$ units/mg) were added, followed by incubation for 25 hr at 37°C. Determination of the extent of glycosylation was made by comparing relative migration of treated and untreated samples on 12.5% SDS–PAGE gels.

3.2.4 Identification of the major SDS–PAGE deglycosylated bands by mass spectrometry

Major deglycosylated bands in the gel were cut out and outsourced to the Proteomics Core Facility, Michael Smith Labs, UBC for partial proteomics analysis. Samples were digested from SDS–PAGE gels with trypsin using a Millipore Montage Zip Plate. Peptide mass fingerprinting was performed by

ESI MS/MS (PE SCIEX API 300 Triple Quad) with a MASCOT database search for protein identification.

3.3 Determination of ovotransferrin antimicrobial activity

3.3.1 Bacterial strains

E. coli EDL933 O157:H7, *E. coli* K–12*, S. aureus* COL and *S. aureus* were individually cultured by the streak plate procedure, in Luria–Bertani (LB) agar (Fisher Scientific) for 24 hr at 37°C.

One colony of each was inoculated into a 3 ml of Brain Heart Infusion (Fisher Scientific) for 24 hr at 37°C, to reach a concentration of 10^9 CFU/ml (two biological repeats). After 24 hr of incubation, the samples were diluted x 1000 with BHI, resulting in a concentration of 10^6 CFU/ml (solution A). Determination of the bacteria concentration was executed by serial dilutions of solution A to 10^{-2}-10^5. One hundred µl of each dilution was plated on LB agar plate (in duplicates), followed by spreading using a sterile loop. The number of colonies was counted after 24 hr at 37°C.

3.3.2 Ovotransferrins preparation

Chicken ovotransferrin: Freeze dried chicken ovotransferrin was purchased from Sigma–Aldrich and a concentration of 20 mg/ml was prepared in deionized water.

Tinamou ovotransferrin: Native ovotransferrin was isolated according to a modified method of Awadé and Efstathiou (1999). Ovotransferrin fractions were collected from the FPLC fractions, concentrated and desalted as described in section 3.2.1. The concentrated fractions were freeze-dried resulting in a white powder and analyzed for purity by SDS–PAGE. A concentration of 20 mg/ml in deionized water was prepared from the freeze dried ovotransferrin. Both chicken and tinamou ovotransferrin solutions were sterilized by passing through a 0.22 µm syringe filter (Amicon).

To obtain the apo form of the protein, freeze–dried tinamou native ovotransferrin and chicken native ovotransferrin were dialyzed against 0.1 M citrate pH 4.5 containing 0.1% EDTA for 24 hr at 4°C, then against an excess of deionized water for two days.

Holo ovotransferrin was prepared from freeze-dried tinamou ovotransferrin and chicken ovotransferrin using a 10 molar excess of Fe^{+3} to ovotransferrin. Ovotransferrins were dialyzed against excess 0.1 M $FeCl_3:6H_2O$ and 50 mM $NaHCO_3$ and then against an excess of deionized water for two days (Ibrahim et al., 1997). An immediate color change to salmon pink corresponding to a distinct peak at ~475 nm was obtained after iron saturation. The apo and holo ovotransferrins were freeze–dried to a fine powder and stored in –20°C for further analysis.

3.3.3 Determination of ovotransferrins bacteriostatic activity by turbidity assay

The antimicrobial activity of ovotransferrin solutions against *E. coli* K–12, *S. aureus*, *E. coli* O157:H7 and *S. aureus* COL in BHI combined with 0, 50 or 100 mM $NaHCO_3$ was determined by measuring the optical density with a microplate reader (BioRad) at 595 nm at 0 hr, after 5 hr and 24 hr of incubation at 37°C. The test wells contained 5 µL of bacterial inoculum and BHI media, water, ovotransferrin and/or $NaHCO_3$ as described in Table 2. Three technical replicates of each of two biological replicates were made for each condition. The composition of the samples used to evaluate the antimicrobial activity against *E. coli* K–12 and *S. aureus* were samples 1–9, for *S. aureus* COL and *E. coli* O157:H7 samples 1–6, 10 and 11. For each set of samples a negative control composed of only distilled water (110 µL) and BHI (80 or 90 µL) in triplicate was used. The negative controls were executed without bacterial inoculums to measure the OD value caused only by the media, as well as to verify that no contamination had occurred in each lane. The positive control of the assay was sample 1, containing only distilled water (110 µL) and BHI (80 or 90 µL) in triplicates, with five µL bacteria.

Table 2 Sample preparation for turbidity assay of ovotransferrins

| Sample | OTF Species | OTF type | Sample properties | | Sample content (µL) | | | |
			OTF (mg/ml)	NaHCO$_3$ (mM)	DW	OTF 20 mg/ml	NaHCO$_3$ 1 M	BHI (2X)
1	Chic, Tin	Apo,Holo,Nat	0	0	110	0	0	90
2	Chic, Tin	Apo,Holo,Nat	0	50	100	0	10	90
3	Chic, Tin	Nat	1	0	100	10	0	90
4	Chic, Tin	Apo,Holo,Nat	10	0	10	100	0	90
5	Chic, Tin	Nat	1	50	90	10	10	90
6	Chic, Tin	Apo,Holo,Nat	10	50	0	100	10	90
7	Chic	Nat	0	100	90	0	20	90
8	Chic	Nat	1	100	80	10	20	90
9	Chic	Nat	10	100	0	100	20	80
10	Chic, Tin	Apo,Holo	5	0	60	50	0	90
11	Chic, Tin	Apo,Holo	5	50	50	50	10	90

*OTF-ovotransferrin; Chic=chicken; Tin=tinamou; Nat=native,
**Each test well contained 5 µL of inoculum

The OD data recorded was normalized as following: For samples without 50 mM NaHCO$_3$, OD was divided by the OD of samples containing media only. Those samples were treated as a relative control for the test samples. For example, the control for samples 3, 4, and 10 was sample 1.

For samples with 50 mM NaHCO$_3$, OD was divided by the OD of samples containing media and 50 mM NaHCO$_3$. Those samples were treated as a relative control to the test samples. For example, the control for samples 5, 6 and 11 was sample 2.

The percent inhibition of *E. coli* O157:H7 and *S. aureus* COL caused by ovotransferrin solutions + 50 mM NaHCO$_3$ was calculated as: % inhibition $= 100 * (1 - \frac{\text{(OD Test Sample} - \text{Negative Control)}}{\text{(OD Positive Control} - \text{Negative Control)}})$

3.3.4 Determination of ovotransferrins bactericidal activity by viability assay

A viability assay was done in order to determine the bactericidal activity of both chicken and tinamou ovotransferrin solutions against *E. coli* O157:H7 and *S. aureus* COL. The number of viable cells was determined by spread plating each sample from the turbidity assay after further dilutions with 0.1 %

peptone. In wells where obvious growth of bacteria was indicated (samples 1–5), four dilutions were made (10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} for *E. coli* and 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} for *S. aureus*).

In wells where no bacteria growth was indicated (sample 6), four dilutions were made (10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}). From each dilution, 100 µl was spread on LBA plate (2 replicates for each concentration) and the number of colonies was counted after incubating plates for 24 hr at 37°C.

3.4 Statistics

3.4.1 Statistical analysis for determination of the antimicrobial activity of native chicken and tinamou ovotransferrins against *E. coli* O157:H7 and *S. aureus* COL.

Two statistical analyses were executed: One compared different conditions within ovotransferrin type for a certain bacteria, for example, a comparison between 1 and 10 mg/ml ovotransferrin of chicken antimicrobial activity against *E. coli* O157:H7. This analysis was executed using a single factor ANOVA (MS Excel 2007) ($p < 0.05$). The second statistical analysis was done in order to compare the same treatment between chicken and tinamou ovotransferrin for a certain bacteria. For example, a comparison between chicken and tinamou 10 mg/ml ovotransferrin antimicrobial activity against *E. coli* O157:H7. For that purpose, a two–tailed t–test was used ($p < 0.05$).

3.4.2 Statistical analysis for determination of the antimicrobial activity of apo and holo chicken and tinamou ovotransferrins against *E. coli* O157:H7.

Statistical analysis was executed by 3 and 4 way ANOVA using Minitab 15.

4. RESULTS

The first results section (Section 4.1) describes the initial characterization of chicken, tinamou, and emu egg whites. The characterization included total protein, sialic acid, ash and iron content determination. The second section (Section 4.2) describes a small scale proteomics analysis of the tinamou egg white proteins and a comparison to the chicken egg white proteins. The last results section (Section 4.3) describes the characterization of the tinamou egg white ovotransferrin antimicrobial activity.

4.1 Chicken, tinamou and emu egg white initial characterization

Chicken, tinamou and emu egg whites were initially characterized by their total protein, sialic acid, ash, and iron content, as well as lysozyme activity (Table 3).

Table 3 Characterization of tinamou, chicken and emu egg white

	Chicken	Tinamou	Emu
Protein (%w/w)	9.5 ± 0.3^a	10.0 ± 0.3^a	6.8 ± 0.5^b
Sialic acid (mg /ml egg white)	1.75 ± 0.05^c	1.06 ± 0.17^b	17.08 ± 0.78^a
Iron (µg /ml egg white)	0.59 ± 0.16^b	0.43 ± 0.02^b	0.61 ± 0.04^a
Ash (%w/w)	0.59 ± 0.14^b	0.68 ± 0.11^a	0.66 ± 0.11^a
Lysozyme activity (units/ml)	34,000	[1]17,000	[2]No lysozyme

Results presented are means ± standard deviation. Statistically different results are labeled with different letters (a,b and c); [1] Fresh tinamou egg white; [2] Maehashi *et al.*, 2010

The egg whites proteins were characterized by SDS–PAGE separation (Fig. 3) and the major proteins were identified by comparing to molecular weight markers and to the literature (Table 4).
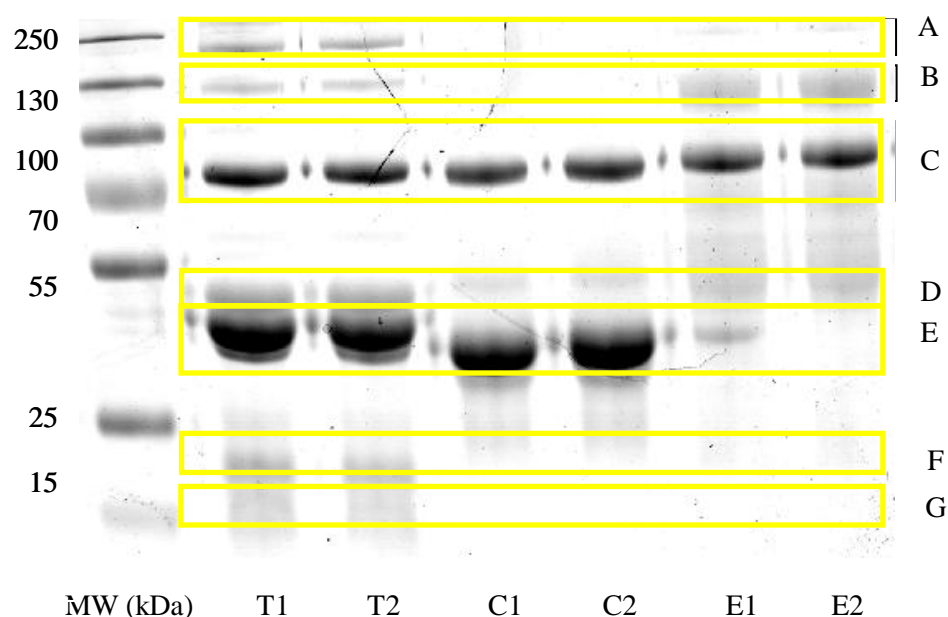
Figure 3 12.5% SDS–PAGE profile of duplicates of tinamou (T1, T2), chicken (C1, C2) and emu (E1, E2) egg white samples. Twenty micrograms of protein were loaded in each lane. Molecular weight was calculated according to bands relative mobility and protein identity was tentatively assigned as specified on table 4.

Table 4 Calculated molecular weights and tentative protein identities of SDS–PAGE bands

	Protein identity	Calculated molecular weights (kDa)of SDS–PAGE bands (Fig. 3)		
		tinamou	chicken	emu
A	Ovomucin	173		
B		144		134
C	Ovotransferrin	86	86	92
D	Ovoglobulin	47	46	
E	Ovalbumin	38	30	
F	Ovomucoid	23		
G	Lysozyme	14		

Tinamou egg white profile contained seven major protein bands, whereas chicken egg white contained three and emu egg white contained two. The molecular weights of those proteins were calculated by the relative mobility (Rf) using a molecular weight marker standard curve (an example for such curve can be seen on Appendix A.1.7). The molecular weight results were compared to literature, for protein identification.

Since ovotransferrin was the common protein of all three egg whites, and it was the main protein of focus throughout the research, the relative ovotransferrin content was determined. The comparison between ovotransferrin contents of egg whites was made using the SDS–PAGE analysis software (Image Quant TL). The average optical densities measured were 0.32, 0.42 and 0.51 OD for chicken, tinamou and emu ovotransferrins, respectively. Comparing the optical densities allowed the quantification of ovotransferrin content differences. According to ratio calculation of the optical densities, tinamou egg white contained approximately 30% more ovotransferrin than chicken. Emu egg white contained approximately 21% more ovotransferrin than tinamou and approximately 60% more than chicken.

4.2 Isolation and identification of the Chilean tinamou major egg white proteins and comparison to the chicken egg white proteins

The identification of tinamou egg white proteins was carried out as a small scale proteomics analysis. The egg white proteins were initially separated by a two steps procedure. The first step was separating the protein to major fractions by anion–exchange FPLC. The second step was separating the major FPLC fractions by SDS–PAGE. Finally, major protein bands identity was confirmed by mass fingerprinting and identification by the MASCOT database.

4.2.1 Separation and identification of the tinamou and chicken egg white proteins

Egg white proteins, with ovomucin removed by precipitation, were separated by anion exchange FPLC. The proteins were eluted by increasing salt concentrations of 0.5 M NaCl, as can be seen by the representative chromatogram in Fig. 4.
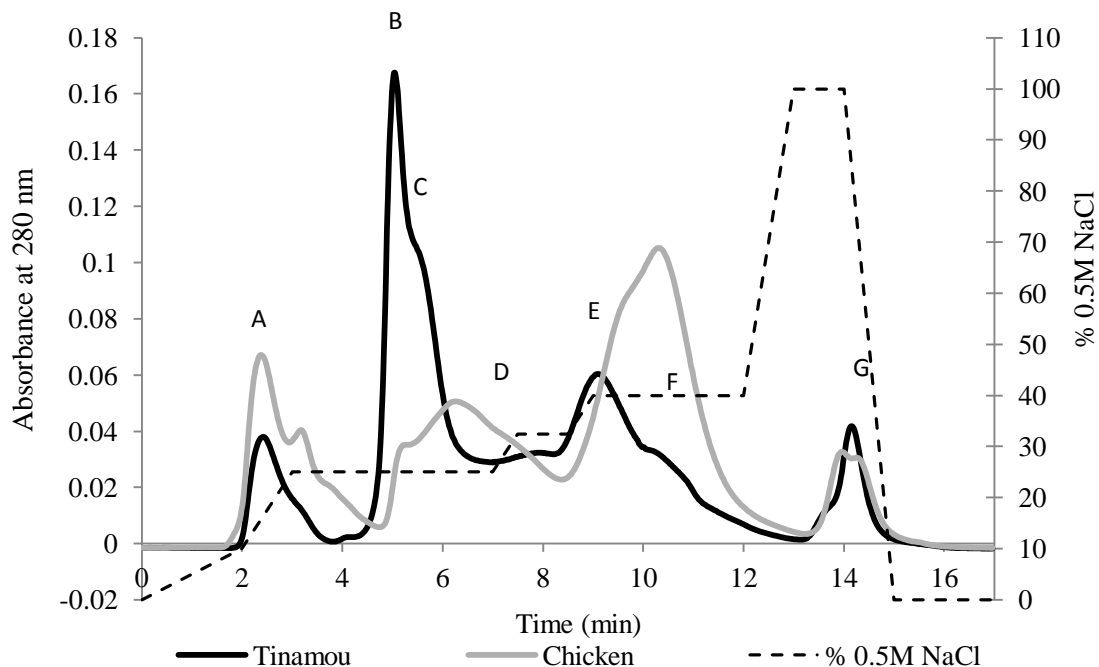
Figure 4 Anion–exchange FPLC of egg white proteins. An aliquot of 500 µl of 10–fold diluted egg white (0.02 M Tris–HCl, pH 9) was separated using a HiTrap Q HP 5/1 column. Fractions A–G were collected and subjected to SDS–PAGE to confirm identity (Fig. 5).

Fractions A–G were concentrated as specified on 3.2.1 and subjected to SDS–PAGE. The SDS–PAGE profile presented a wide range of relative molecular masses, as shown in Fig. 5.
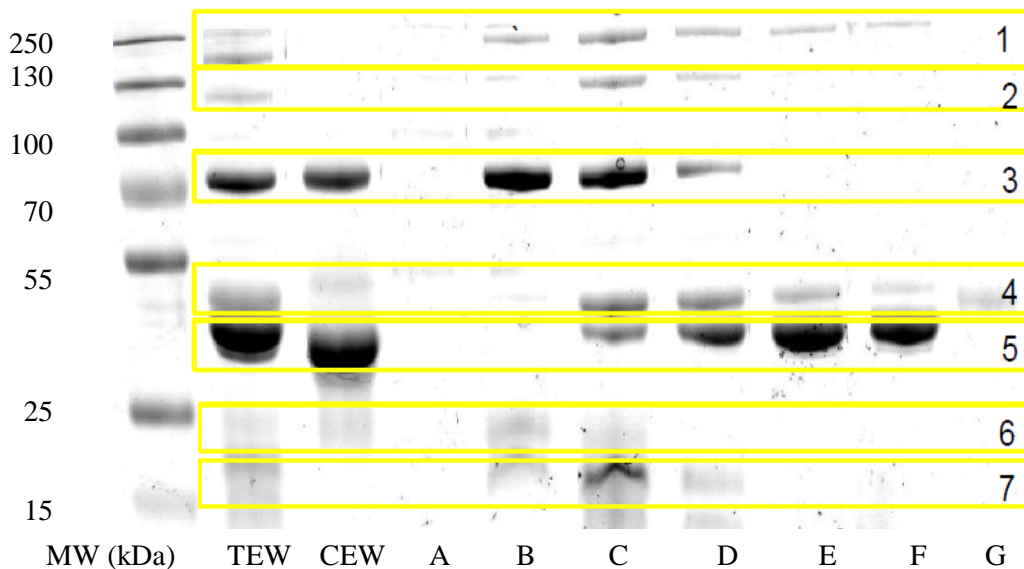
Figure 5 12.5% SDS–PAGE of tinamou and chicken egg white (10 mg/ml) and FPLC tinamou egg white fractions A–G. Injection volume was 10 µL for samples and 5 µL for marker. Molecular weight was calculated according to the relative mobility, and proteins were tentatively identified by comparing to the literature: (1) Ovomucin, 187 kDa; (2) 150 kDa; (3) Ovotransferrin, 89 kDa; (4) Ovoglobulin, 51 kDa; (5) Ovalbumin, 42 kDa; (6) Ovomucoid, 21kDa; (7) Lysozyme, 12 kDa.

4.2.3 Deglycosylation of tinamou egg white proteins by PNGase–F

Fractions C, D and E were further concentrated and treaded with PNGase–F in order to deglycosylate the proteins. Deglycosylation was an essential step prior peptide digestion and amino acid mass sequencing. A comparison of fractions migration on SDS–PAGE before and after treatment is shown in Fig. 6.
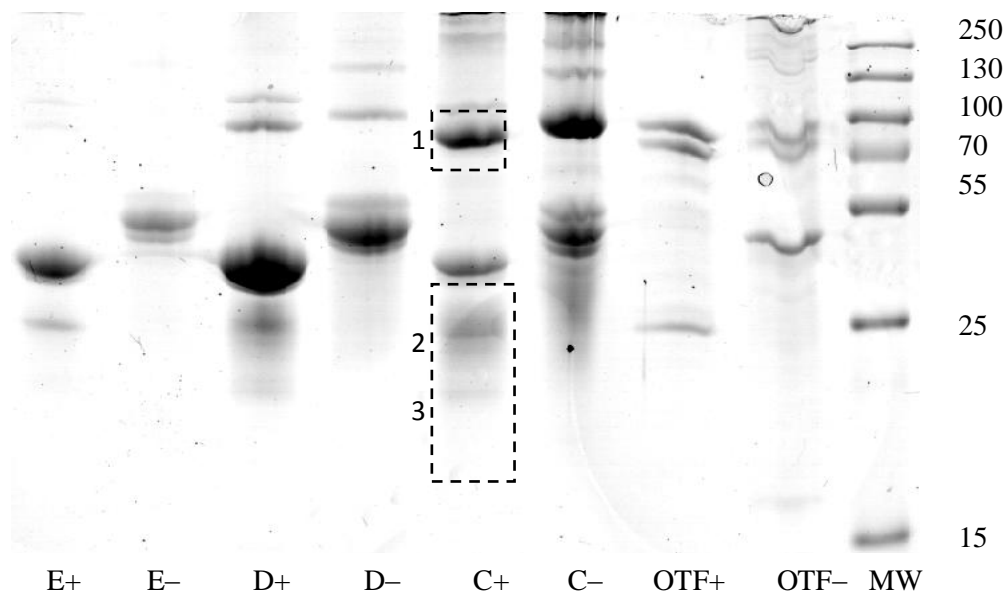
Figure 6 12.5% SDS–PAGE of PNGase–F treated fractions C, D, E and 5 mg/ml chicken OTF standard. Fractions before (–) and after (+) deglycosylation. The dashed fragments were cut out of the gel and sent for mass spectral analysis. Bands were identified as (1) ovotransferrin; (2) ovomucoid; and (3) lysozyme by peptide sequence as shown in Figure 7.

Glycosylation content of the ovotransferrin and ovalbumin was measured by the relative migration distance of the proteins band on the SDS–PAGE (Appendix A.1.7).

PNGase–F treatment caused a MW decrease of 5 and 7 kDa in the tinamou ovotransferrin and ovalbumin bands, respectively, confirming that these proteins are N–linked glycoproteins. Tinamou ovotransferrin and ovalbumin comprise of 6% and 15% glycan residues, respectively.

4.2.4 Identification of tinamou egg white proteins by mass spectrometry

The deglycosylated bands (lane C+, Fig. 6) were cut from the gel and digested with trypsin (theoretical digestion map is shown in Appendix A.1.8). Peptide mass fingerprinting was performed by ESI MS/MS with a MASCOT database search for protein identification, as seen on Fig. 7. An ion score (IS) was assigned to each peptide identification. Ion score was calculated as 10*Log(P), where P is the probability that the observed match is a random event. Individual ion scores >49 indicate identity or extensive homology (p<0.05). Protein scores are the summed scores for the individual peptides, e.g. peptide masses and peptide fragment ion masses, for all peptides matching a given protein.

Ovotransferrin (79,551 Da) PS=84

```
191-250   RQCKGDPKTK  CARNAPYSGY  SGAFHCLKDG  KGDVAFVKHT  TVNENAPDQK  DEYELLCLDG
251-310   SRQPVDNYKT  CNWARVAAHA  VVARDDNKVE  DIWSFLSKAQ  SDFGVDTKSD  FHLFGPPGKK
311-370   DPVLKDLLFK  DSAIMLKRVP  SLMDSQLYLG  FEYYSAIQSM  RKDQLTPSPR  ENRIQWCAVG
371-430   KDEKSKCDRW  SVVSNGDVEC  TVVDETKDCI  IKIMKGEADA  VALDGGLVYT  AGVCGLVPVM
431-490   AERYDDESQC  SKTDERPASY  FAVAVARKDS  NVNWNNLKGK  KSCHTAVGRT  AGWVIPMGLI
491-550   HNRTGTCNFD  EYFSEGCAPG  SPPNSRLCQL  CQGSGGIPPE  KCVASSHEKY  FGYTGALRCL
551-610   VEKGDVAFIQ  HSTVEENTGG  KNKADWAKNL  QMDDFELLCT  DGRRANVMDY  RECNLAEVPT
611-670   HAVVVRPEKA  NKIRDLLERQ  EKRFGVNGSE  KSKFMMFESQ  NKDLLFKDLT  KCLFKVREGT
```

Ovomucoid Fragment (5,901 Da) PS=313

```
151-210   CRKELAAVSV  DCSEYPKPDC  TAEDRPLCGS  DNKTYGNKCN  FCNAVVESNG  TLTLSHFGKC
```

Lysozyme C (16,228 Da) PS=179

```
1-60      MRSLLILVLC  FLPLAALGKV  FGRCELAAAM  KRHGLDNYRG  YSLGNWVCAA  KFESNFNTQA
61-120    TNRNTDGSTD  YGILQINSRW  WCNDGRTPGS  RNLCNIPCSA  LLSSDITASV  NCAKKIVSDG
```

Figure 7 Sequence homology of Chilean tinamou egg white proteins with chicken egg white proteins. The deglycosylated proteins (Band C+, Fig. 4) were subjected to mass spectrometry using ESI MS/MS and fingerprinting using the MASCOT database. The underlined peptides are homologous with chicken protein sequences, confirming the identity of lysozyme C, ovotransferrin and ovomucoid. PS=protein scores

The peptide fingerprinting of Chilean tinamou egg white proteins confirmed the identities of ovotransferrin and lysozyme C, by comparing to peptides of chicken egg white proteins. Ovomucoid identity was compared to peptides in the MASCOT database, and a short Chilean tinamou egg white ovomucoid peptide was in the database; therefore the identification was by comparing to that sequence, resulting in a high ion score.

4.3 Determination of ovotransferrin antimicrobial activity

The second aim of the study was to determine the antimicrobial activity of chicken and tinamou ovotransferrin against pathogenic *E. coli* O157:H7 and *S. aureus* COL. As a preliminary step, the bacteriostatic activity of commercial chicken ovotransferrin was tested against non–pathogenic *E. coli* K-12 and *S. aureus* (Section 4.3.1). Following that, bacteriostatic and bactericidal activity of native tinamou and chicken (1 and 10 mg/ml) ovotransferrins was determined against pathogenic *E. coli* O157:H7 and *S. aureus* COL, respectively (Section 4.3.2). The last step was determining the bacteriostatic and bactericidal activity of apo and holo tinamou and chicken (5 and 10 mg/ml) ovotransferrins against *E. coli* O157:H7 (Section 4.3.3).

4.3.1 Determination of the bacteriostatic activity of commercial chicken ovotransferrin against *E. coli* K-12 and *S. aureus*

The bacteriostatic activity of commercial chicken ovotransferrin was tested against non–pathogenic *E. coli* K-12 and *S. aureus* as an initial step in order to establish the effective concentration range of the protein and bicarbonate (Appendix A.2.1). Two chicken ovotransferrin concentrations (1 and 10 mg/ml), combined with three different bicarbonate concentration (0, 50 and 100 mM) were examined. A concentration of 50 mM $NaHCO_3$ on its own did not inhibit the growth of *E. coli* K-12 and *S. aureus*, however, a concentration of 100 mM $NaHCO_3$ was bacteriostatic towards both *E. coli* K-12 and *S. aureus*. From these observations, a concentration of 50 mM $NaHCO_3$ was selected. The concentrations of ovotransferrins were selected according to references describing the antimicrobial

activity of chicken ovotransferrins (Ko *et al*., 2008; Valenti *et al*., 1980). Due to low availability of tinamou ovotransferrin, the concentrations that were chosen were 1 and 10 mg/ml ovotransferrins.

4.3.2 Determination of the antimicrobial activity of native chicken and tinamou ovotransferrins against *E. coli* O157:H7 and *S. aureus COL*

The bacteriostatic activity against *E. coli* O157:H7 of native ovotransferrin solutions can be seen in Fig. 8.
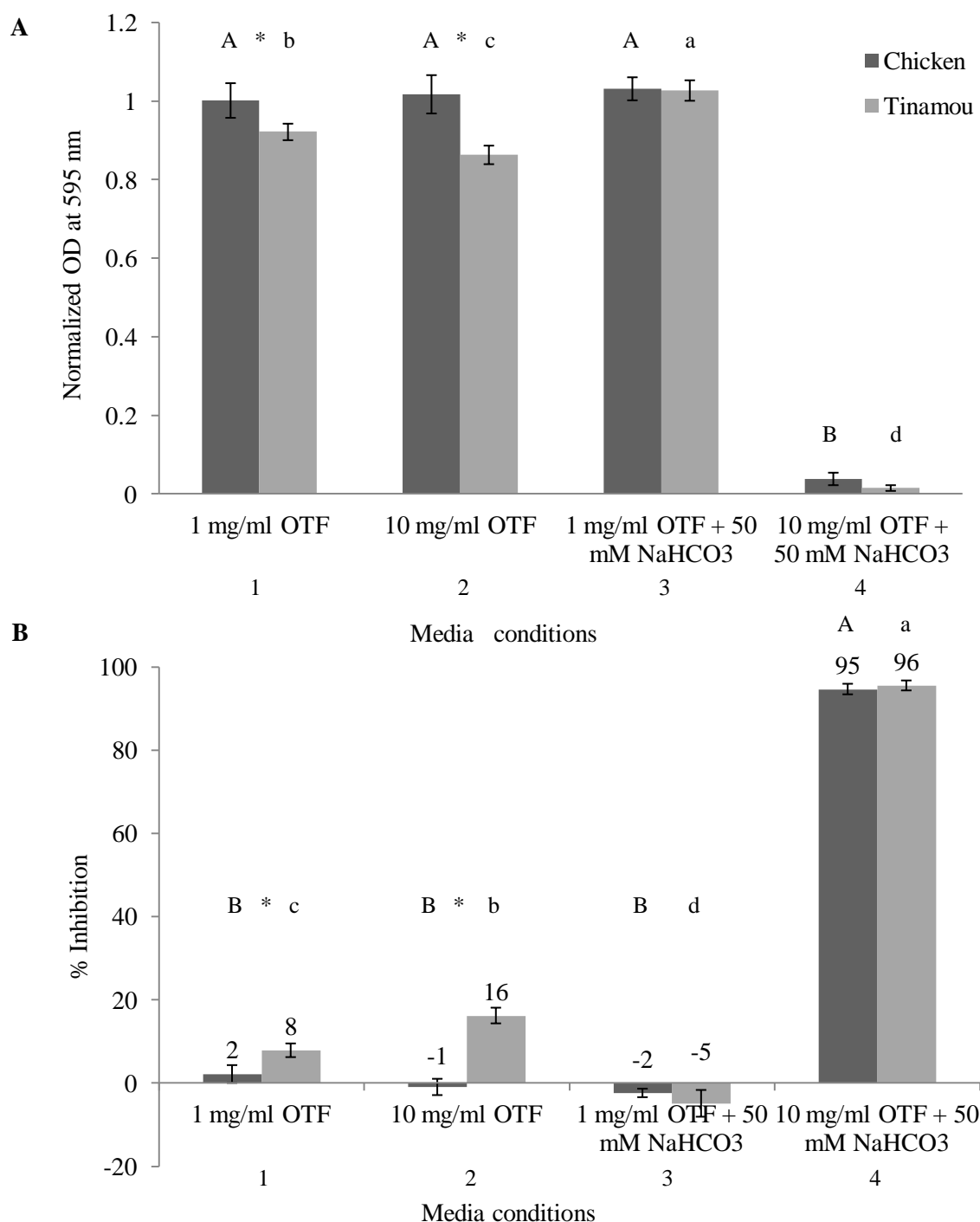
Figure 8 The bacteriostatic activity of ovotransferrins against *E. coli* O157:H7 after incubation for 24 hr at 37°C. A) Normalized OD at 595 nm; **B)** % inhibition by ovotransferrin (OTF). The OD for samples 1 and 2 was normalized by dividing the OD by the OD of a control containing media only. OD for samples 3 and 4 was normalized by dividing the OD by the OD of a control containing media + 50 mM NaHCO₃. Capital letters denote statistical significance of chicken OTF samples and low case letters for statistical significance of tinamou OTF samples. Asterisks denote statistical significance between chicken and tinamou OTF samples under the same condition (p<0.05).

The main trend observed was that both 10 mg/ml chicken and tinamou ovotransferrin combined with 50 mM $NaHCO_3$ had the greatest bacteriostatic effect against *E. coli* O157:H7, with inhibition of 95±1% and 96±1%, respectively.

Tinamou ovotransferrin samples exhibited minor bacteriostatic activity against *E. coli* O157:H7 without 50 mM $NaHCO_3$; 8±2% and 16±2% inhibition were observed for 1 and 10 mg/ml ovotransferrin, respectively. However, this was greater than the effect of chicken ovotransferrin, which had no inhibition at 1 and 10 mg/ml.

Bactericidal activity of ovotransferrin solutions against *E. coli* O157:H7 was determined by a viability assay and presented as log reduction (Table 5).

Table 5 Bactericidal activity of native ovotransferrins against *E. coli* O157:H7

Sample*	Log reduction (CFU/ml)	
	Chicken	Tinamou
#3 – 1 mg/ml + 50 mM $NaHCO_3$	1	1
#4 – 10 mg/ml + 50 mM $NaHCO_3$	9	9

*Samples 1–2, without $NaHCO_3$, did not exhibit a bactericidal effect.

Only ovotransferrin samples containing 50 mM $NaHCO_3$ were bactericidal against *E. coli* O157:H7, and no bacterial growth was observed for both chicken and tinamou ovotransferrin at 10 mg/ml with 50 mM $NaHCO_3$. Native ovotransferrins were more inhibitory against *S. aureus* COL at the lower concentration of 1 mg/ml with 50 mM $NaHCO_3$, as shown in Fig. 9 and Table 6.
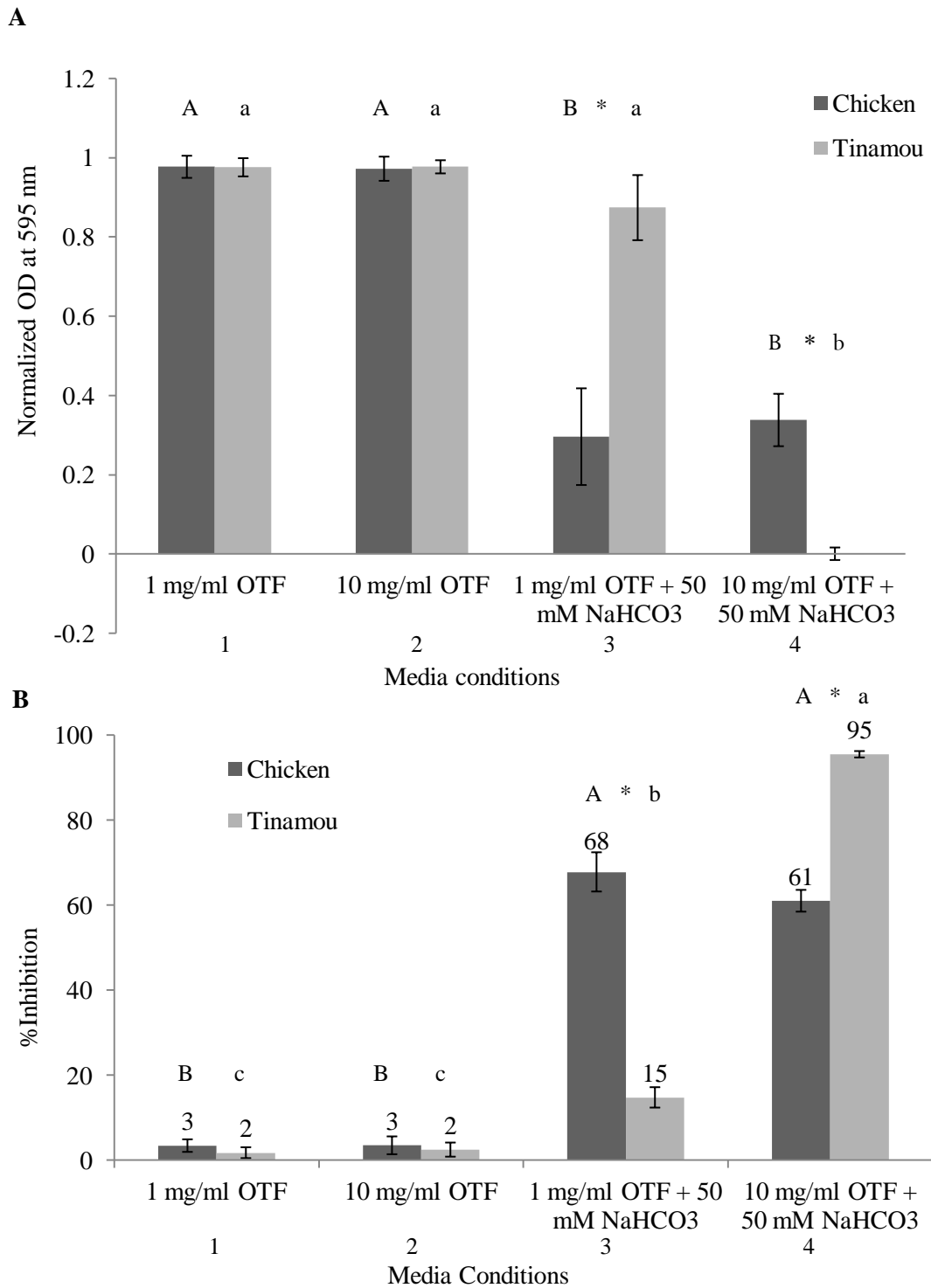
Figure 9 The bacteriostatic activity ovotransferrin (OTF) against *S. aureus* COL after incubation for 24 hr at 37°C. **A)** Normalized OD at 595 nm. **B) %** Inhibition by OTF solutions. The OD for samples 1 and 2 was normalized by dividing the OD by the OD of a control containing media only. OD for samples 3 and 4 was normalized by dividing the OD by the OD of a control containing media + 50 mM $NaHCO_3$. Capital letters denote statistical significance of chicken OTF samples and low case letters for statistical significance of tinamou OTF samples. Asterisks denote statistical significance between chicken and tinamou OTF samples under the same condition ($p < 0.05$)

Ovotransferrin samples combined with 50 mM $NaHCO_3$ inhibited bacterial growth more significantly than ovotransferrin samples without 50 mM $NaHCO_3$. Among 50 mM $NaHCO_3$ treated samples; it can be observed that 10 mg/ml tinamou ovotransferrin inhibited the bacterial growth more than 1 mg/ml tinamou ovotransferrin, whereas inhibition by chicken ovotransferrin was the same for both concentrations. The most effective combination was 10 mg/ml tinamou ovotransferrin with 50 mM $NaHCO_3$ which resulted in $95\pm1\%$ inhibition of *S. aureus* COL. The 1 and 10 mg/ml chicken ovotransferrins with 50 mM $NaHCO_3$ resulted in $61\pm3\%$ and $68\pm5\%$ inhibition, respectively. Bactericidal activity of ovotransferrin solutions against *S. aureus* COL was determined by a viability assay and presented as log reduction (Table 6).

Table 6 Bactericidal activity of native ovotransferrins against *S. aureus* COL

	Log reduction (CFU/ml)	
Sample*	Chicken	Tinamou
#3 – 1 mg/ml + 50 mM $NaHCO_3$	3	1
#4 – 10 mg/ml + 50 mM $NaHCO_3$	9	9

*Samples 1–2, without $NaHCO_3$ did not exhibit a bactericidal effect.

Only ovotransferrin samples containing 50 mM $NaHCO_3$ were bactericidal against *S. aureus* COL. No bacterial growth was observed for both chicken and tinamou ovotransferrins at 10 mg/ml with 50 mM $NaHCO_3$.

4.3.3 Determination of the antimicrobial activity of apo and holo chicken and tinamou ovotransferrins against *E. coli* O157:H7

Native ovotransferrins were chemically manipulated to the apo and holo forms. An immediate color change to salmon pink corresponding to a distinct peak at ~475 nm was obtained after iron saturation.
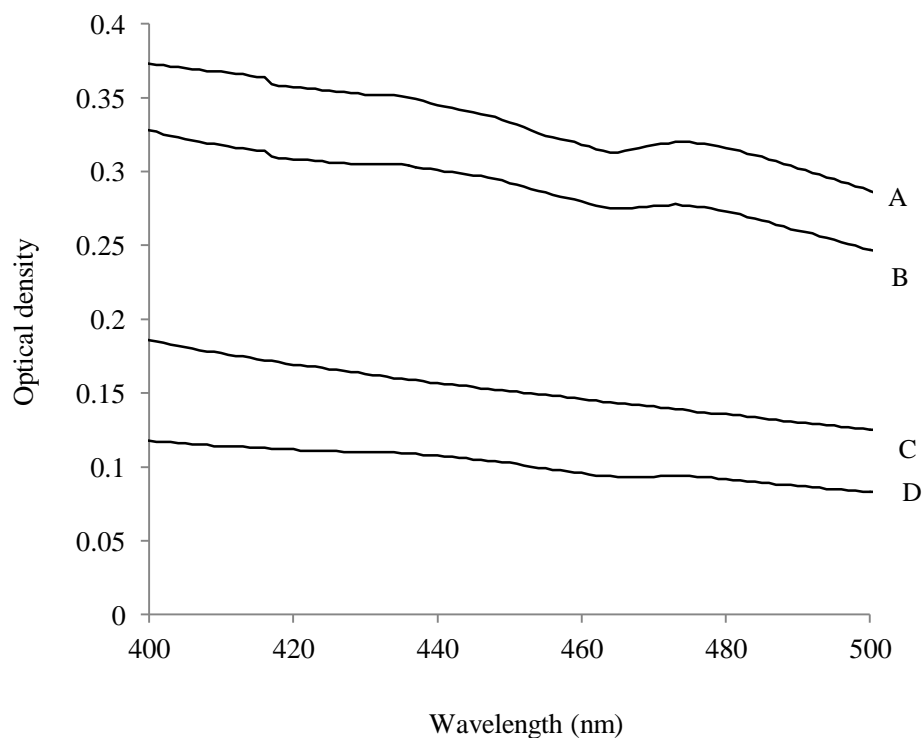
Figure 10 Absorption spectra of apo and holo chicken and tinamou ovotransferrins. A) 5.6 mg/ml holo chicken. Maximum absorption of 0.32 was observed at 475 nm; B) 4.9 mg/ml holo tinamou. Maximum absorption of 0.28 was observed at 473nm; C) 2.9 mg/ml apo chicken; D) 2.1 mg/ml apo tinamou.

The bacteriostatic activity against *E. coli* O157:H7 of apo and holo ovotransferrin solutions (5 or 10 mg/ml) with or without 50 mM NaHCO$_3$ was determined (Fig. 11).
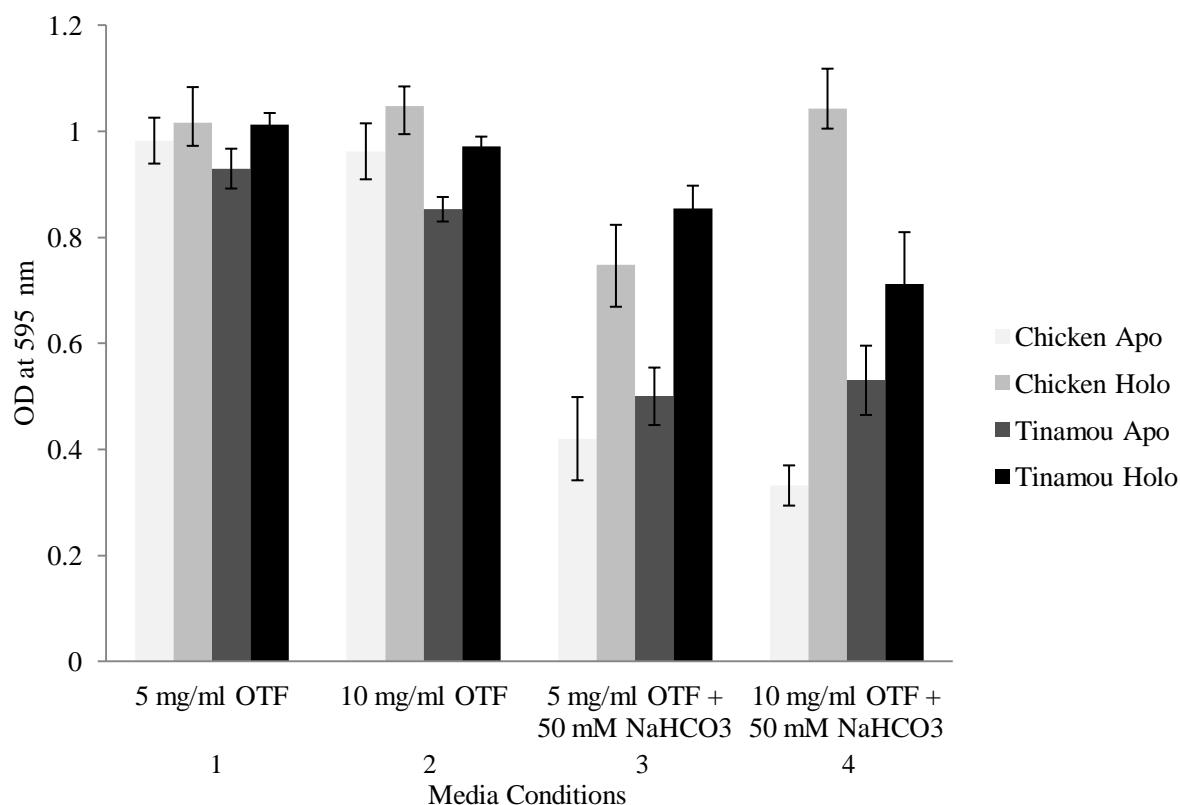
Figure 11 The bacteriostatic activity of ovotransferrins against *E. coli* O157:H7 after incubation for 24 hr at 37°C. OTF=ovotransferrin. The OD for samples 1 and 2 was normalized by dividing the OD by the OD of a control containing media only. OD for samples 3 and 4 was normalized by dividing the OD by the OD of a control containing media + 50 mM $NaHCO_3$.

Ovotransferrin at both concentrations, combined with 50 mM $NaHCO_3$, inhibited the bacterial growth more than ovotransferrin samples without 50 mM $NaHCO_3$. In the presence of $NaHCO_3$, apo ovotransferrins inhibited the bacterial growth more significantly than holo ovotransferrins, with chicken apo ovotransferrin exhibiting the greatest inhibition.

Only ovotransferrin samples containing 50 mM $NaHCO_3$ exhibited bactericidal activity against *E. coli* O157:H7, as determined by a viability assay with a 1 log (CFU/ml) reduction (Table 7).

Table 7 Bactericidal activity of apo ovotransferrins against *E. coli* O157:H7

	Log reduction (CFU/ml)	
Sample*	Chicken	Tinamou
#3 – 5 mg/ml + 50 mM $NaHCO_3$	1	1
#4 – 10 mg/ml + 50 mM $NaHCO_3$	1	1

*Samples 1–2, without $NaHCO_3$, and holo OTF samples, did not exhibit a bactericidal effect.

The bacteriostatic activity results were initially analysed by a 4 way ANOVA (Appendix A.2.4), and the main effects and their interactions were examined. The analysis demonstrated that the main effect of species, $NaHCO_3$, and iron saturation was significant (Table 8).

Table 8 Four–way analysis of variance for ovotransferrins bacteriostatic activity against *E. coli* O157:H7

	Degrees of Freedom	P–value
OTF Species (chicken/tinamou)	1	0.033
OTF type (apo/holo)	1	0.000
OTF concentration (5/10 mg/ml)	1	0.116
50 mM $NaHCO_3$ (+/–)	1	0.000

*OTF=Ovotransferrin

There was no significant difference between 5 and 10 mg/ml ovotransferrin; therefore, a 3 way ANOVA (Appendix A.2.4) was conducted with the significant factors of 5 mg/ml ovotransferrin (Fig. 12).
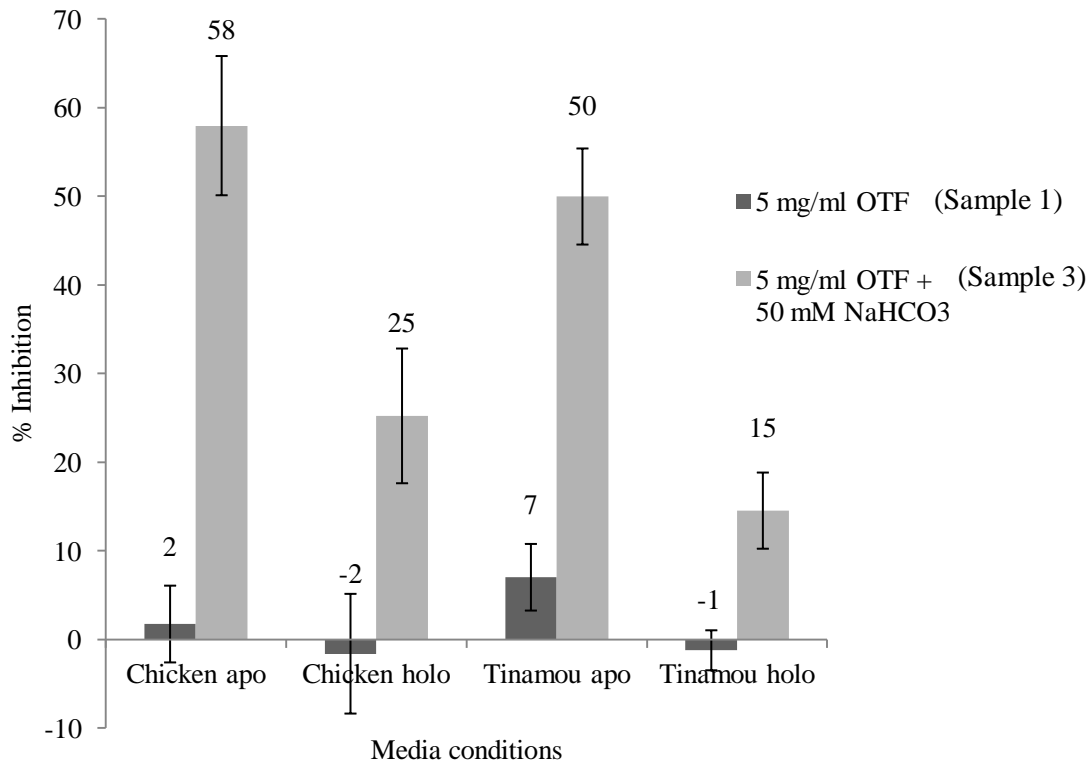
Figure 12 The bacteriostatic activity of 5 mg/ml ovotransferrins (OTF) against *E. coli* O157:H7 after incubation for 24 hr at 37°C in 4 different conditions. The OD for samples assayed without 50 mM NaHCO$_3$ were normalized by dividing the OD by the OD of a control containing media only. The OD for samples assayed with 50 mM NaHCO$_3$ were normalized by dividing the OD by the OD of a control containing media + 50 mM NaHCO$_3$.

Three–way ANOVA examined the main effects of species, ovotransferrin type, 50 mM $NaHCO_3$ and their two level interactions (Table 9).

Table 9 Three–way analysis of variance for ovotransferrins bacteriostatic activity against *E. coli* O157:H7

	Degrees of Freedom	P–value
Species (chicken/tinamou)	1	0.048
OTF type (apo/holo)	1	0.000
50 mM $NaHCO_3$ (+/−)	1	0.000
Species*OTF type	1	0.243
Species*50 mM $NaHCO_3$	1	0.000
OTF type*50 mM $NaHCO_3$	1	0.000

*OTF=Ovotransferrin

This analysis demonstrated that the main effect of species, $NaHCO_3$, and iron saturation was significant. There was no interaction effect between ovotransferrin species and ovotransferrin type. Chicken apo and holo ovotransferrins had similar inhibition levels on average, as tinamou apo and holo ovotransferrins.

The results were clearly different between ovotransferrins with and without 50 mM $NaHCO_3$, regardless of ovotransferrin type and species. Ovotransferrin samples without 50 mM $NaHCO_3$ had no inhibition against *E. coli* O157:H7, except for tinamou apo, which exhibited a minor level of inhibition of 7±4 %. Both apo chicken and tinamou ovotransferrins had a significant bacteriostatic effect against *E. coli* O157:H7 of 58±8 % and 50±5 % inhibition, respectively. Both holo chicken and tinamou ovotransferrins had a lower bacteriostatic effect against *E. coli* O157:H7 of 25±8 % and 15±4 % inhibition, respectively.

5. DISCUSSION

The first aim of this study was to identify the major antimicrobial components of the Chilean tinamou egg white and to compare them to the chicken and emu major egg white antimicrobial components. Since tinamous are classified as a ratite species, the hypothesis was that more similarities will be observed between Chilean tinamou and emu egg whites, rather than between Chilean tinamou and chicken egg whites. In order to achieve this hypothesis aim, tinamou, chicken and emu egg whites were characterized for total protein, sialic acid, ash and iron content (Section 5.1). Following that, a small scale proteomics analysis of the tinamou egg white proteins was conducted, including the separation and identification of the tinamou egg white proteins, and further a comparison to the chicken egg white proteins (Section 5.2).

The second aim of this study was to characterize and compare the antimicrobial activity of tinamou ovotransferrin to chicken ovotransferrin against two foodborne pathogens, *E. coli* O157:H7 and *S. aureus* COL. The hypothesis was that tinamou egg white may have high quantities of iron saturated ovotransferrin and therefore can serve as an excellent source of a natural antimicrobial agent. Additionally, it was hypothesised that ovotransferrin is antimicrobial regardless of iron saturation state. The antimicrobial activity of native, apo and holo chicken and tinamou ovotransferrin *E. coli* O157:H7 and *S. aureus* COL was determined by turbidity and viability assays (Section 5.3).

5.1 Egg whites characterization

Tinamou, chicken and emu egg whites were initially characterized by their total protein, sialic acid, ash and iron content as specified in Table 3.

5.1.1 Determination of egg whites and FPLC fractions protein content

The protein content of tinamou, chicken and emu egg whites and FPLC tinamou egg white fractions was initially determined by the Bradford protein assay (Bradford, 1976), a sensitive colorimetric method used to measure the concentration of proteins in a solution. Samples were treated with the protein assay dye reagent (Coomassie Brilliant Blue), which changed its color in response to various

concentrations of protein. This assay is based on the principle that the dye shifts from 465 nm to 595 nm when binding to basic amino acid residues, especially arginine. The absorbance of the samples measured at 595 nm is relative to the protein concentration.

Though the Bradford assay confirmed that tinamou FPLC fractions were concentrated by approximately 10 fold after filtration (Appendix A.1.1), results for total protein content in egg whites were inconclusive. Calculated protein concentration varied considerably depending on the dilution used. That may be due to egg white low solubility, and that this method involves very low concentrations. Additionally, the Bradford method is usually more efficient when proteins comprise mostly of basic acid residues. It is important to mention that this experiment was repeated thrice. This is why a second method was used, the Kjeldahl method (AOAC official method 932.08, Nitrogen in liquid eggs) which measures the total nitrogen of a sample ($N \times 6.25$).

The first step of the Kjeldahl method involved heating egg white samples with sulfuric acid, which caused a complete decomposition of the sample, liberating reduced nitrogen as ammonium sulfate that further was converted to ammonia. The amount of ammonia equaled the amount of nitrogen present in the sample and was determined by back titration.

No significant difference between tinamou and chicken egg white total protein content was observed ($p>0.05$), whereas significantly lower protein content (%w/w) was observed for emu egg white ($6.8\pm0.5\%$) compared to tinamou ($10.0\pm0.3\%$) and chicken ($9.5\pm0.3\%$).

Though recent phylogenetic analyses placed tinamous within ratites, which include emu (Harshmann, 2008), tinamou egg white protein concentration was most similar to the chicken egg white, which classifies only remotely to tinamou.

The Chilean tinamou egg white protein content has not been previously determined; however, chicken egg white and emu egg white protein content have been studied before. According to United States Department of Agriculture (USDA) fresh egg white protein concentration is 11.0 %w/w egg white. The emu egg white protein content was determined before by the Kjeldahl method ($N \times 6.25$): Koga (1969) determined 8.08 (%w/w) and Takeuchi and Nagashima (2010) determined 8.9 (%w/w).

Though those reported results for emu are higher, the observed trend was the same with higher protein concentration for chicken egg white rather than emu egg white.

In chicken eggs, the egg yolk and egg white comprises a 1:2 ratio. A relative proportion was observed in tinamou eggs. In emu egg, the proportion is 1:1. It is possible that the protein content distribution is affected by the egg white to yolk ratio. Though emu egg white contains less protein per egg white, the ratio of protein content to the entire egg is higher for emu.

5.1.2 Determination of sialic acid content in egg whites

Sialic acid is a carboxylated sugar which serves as part of large carbohydrates such as glycoproteins. The most common type of sialic acid found in avian egg white is the N–acetylneuraminic acid (Nakano *et al.*, 1994). Sialic acid contributes to cell adhesiveness and enzyme inhibition, and therefore can serve as a natural antimicrobial agent (Reutter *et al.*, 1982). Sialic acid content of tinamou, chicken and emu egg white was determined by the Warren colorimetric method (1959), with 1–propanol as the final extracting solvent instead of cyclohexanone. The resulting chromophore absorbance was read in 549 nm and sialic acid content was calculated from a standard curve equation (Appendix A.1.4).

Tinamou, chicken and emu egg white samples were significantly different ($p < 0.05$) in sialic acid content. Moreover, the sialic acid content (mg sialic acid/ml egg white) of emu egg white (17.08±0.78) was 10 fold higher than chicken egg white (1.75±0.05) and tinamou egg white (1.06±0.17).

Chicken and emu egg white sialic acid content was reported before in few studies. Nakano *et al.* (1994) used the 1–propanol extraction method, and found 0.22 mg sialic acid/ml chicken egg white. Feeney *et al.* (1960) used the original cyclohexanone extraction method, and found that chicken egg white and emu egg white contain 2.9 and 37 mg sialic acid/ml egg white, respectively. Though our results do not confirm the literature values, the same ten-fold difference between chicken egg white and emu egg white was obtained. The Chilean tinamou egg white sialic acid content was not reported

previously. According to our results, once again, tinamou egg white was more similar to chicken, rather than their sister ratite group, the emu.

It would be interesting to determine the sialic acid content in a more advanced way such as an anion–exchange technique in combination with adsorption chromatography on cellulose powder (Schauer, 2000).

5.1.3 Determination of total mineral content in egg whites

Dry ashing method determines the total egg white mineral content, such as Ca, Mg, Na, K, Fe, Zn, Cu and Mn. The iron concentration and its availability increases, by removing the organic matter, throughout the burning of the sample; therefore dry ashing is an important first step in order to determine the actual iron content.

There was no significant difference (p>0.05) between tinamou (0.68±0.11), chicken (0.59±0.14) and emu (0.66±0.11) (%w/w) egg white mineral content. The data regarding chicken egg white agrees with the literature value of 0.70% (w/w) ash (Health Canada, 2010) or 0.63±0.03% (w/w) ash (USDA nutrient database). Tinamou and emu egg white ash content has not been previously reported.

5.1.4 Determination of iron content in egg whites

The ferrozine assay is a colorimetric method that determines the total iron content of a sample. Iron concentration in tinamou egg white, chicken egg white and emu egg white was determined by the ferrozine assay as μg iron/ml egg white (adapted from Lysionek *et al.*, 1998). There was no significant difference (p>0.05) between tinamou (0.43±0.02) and chicken (0.59±0.16) egg white iron content whereas iron concentration in emu (0.61±0.04) egg white was higher.

Results for chicken are in agreement with Health Canada database, reported as 0.06 mg iron/100 g egg white (Health Canada, 2010). The method used was not specified, however the USDA database specifies that atomic absorption (AOAC 985.35) and inductively coupled plasma emission spectrophotometry (AOAC 984.27) was used (USDA Nutrient Database for Standard Reference, Release 17, page 27, NDB 01124);   33.4 g fresh chicken egg white contains 0.03 mg iron which is

equivalent to 0.92 µg iron/ml. Tinamou and emu egg white iron content has not been previously reported.

Chicken egg white ovotransferrin was reported to appear as iron–free in its natural form, as one of the chicken egg white proteins (Feeney *et al.*, 1960). Since tinamou egg white ovotransferrin content was found to be approximately 30% more than chicken egg white ovotransferrin, for the same iron concentration, there is a higher OTF/iron ratio in tinamou egg white than in chicken egg white.

5.2 Isolation and identification of the Chilean tinamou major egg white proteins

As part of the first aim of this study, the major proteins of the Chilean tinamou egg white were identified and to compared to the chicken and emu major egg white proteins. The identification of the major tinamou egg white proteins was carried out as a small scale proteomics analysis. Since chicken egg white proteomics has proven to be a powerful protein characterization tool, proteomics analysis was chosen as a key strategy. The recent application of high–throughput proteomics to analyze chicken egg white proteins has dramatically increased available knowledge regarding the number and nature of egg proteins. Desert *et al.,* (2001) were first to conduct a proteome–level investigation of chicken which led to the characterization of small acidic proteins. Since then, there have been several successful attempts to characterize the chicken egg white proteome, with the most recent analysis preformed by Mann and Mann (2011), identifying 158 egg white proteins.

Proteomics analysis of the tinamou egg white was performed by a three steps procedure: First, egg whites were separated by anion–exchange FPLC to major fractions (Fig. 4), and further fractionated by SDS–PAGE (Fig. 5). In traditional proteomics analysis, the proteins are first separated by charge and then size using 2–D gel electrophoresis. In this research, anion–exchange FPLC was used, followed by SDS–PAGE separation. Both methods achieve the same principle of separation according to charge and molecular weight. The last step was mass spectrometry and peptide fingerprinting for specific protein identification using the MASCOT database (Fig. 7).

Anion–exchange FPLC protein separation was according to the proteins charge in a Tris–HCl pH 9.0 buffer. In that condition, most of the egg white proteins were negatively charged as their isoelectric point is less than 9. The proteins were eluted by increasing salt concentrations of 0.5 M NaCl, in the order of decreasing isoelectric points, excluding lysozyme and avidin (Fraction A). Each FPLC fraction was further subjected to a second separation by a 12.5 % Coomassie stained SDS–PAGE. The proteins migrated through the gel according to their molecular weight, resulting in a better separation resolution than FPLC solely. The final analysis step was mass spectrometry analysis. The desired SDS–PAGE bands were deglycosylated, cleaved out of the gel and outsourced for mass spectroscopic analysis followed by fingerprinting using the MASCOT database.

Deglycosylation is an essential step prior to peptide digestion and amino acid mass sequencing. Following deglycosylation with PNGase-F, desired bands were digested with trypsin and subjected to peptide fingerprinting by tandem mass spectrometry. The peptide fragments obtained were processed and analyzed with suitable computer programs. These programs performed an *in silico* digestion of all sequences in the MASCOT database and compare the theoretical spectra to the experimental spectra and assign to each of them a score. If theoretical and experimental spectra compared favorably and if the mass of the intact peptide was measured with high accuracy, the combined data result in a statistically significant score indicating reliable peptide identification. This kind of high–throughput proteome analysis critically depends on a database which should be as complete as possible, such as the one provided by chicken genome sequence analysis. Since no database of tinamou egg white proteome exists, it was interesting to see that tinamou egg white protein could be identified using chicken database, despite the fact that they are different species.

The discussion will focus on the major identified egg white proteins: lysozyme, ovotransferrin, ovalbumin and ovoglobulin, ovomucoid, ovomucin and unidentified high MW proteins of 144 kDa and 133 kDa for tinamou and emu egg white, respectively,

5.2.1 Lysozyme

Lysozyme is one of the major antimicrobial egg white proteins, present in various avian species (Feeney *et al.*, 1960). The most studied avian lysozyme is chicken egg white lysozyme, a 14 kDa protein with an isoelectric point of 10.7 (Mine, 1995).

As part of the tinamou egg white proteins characterization, lysozyme was compared to chicken and emu lysozyme. Chicken and tinamou egg whites were initially separated by anion–exchange FPLC (Fig. 4). Due to its high isoelectric point value of 10.7, lysozyme has a positive charge at pH 9, consequently it is not bound to the column. Avidin is another minor protein in egg white that has a high isoelectric point (pI=10.1); since avidin content of chicken egg white is very low, 0.05%, the discussion focuses on lysozyme. Under the chromatographic conditions, the electrical charge of lysozyme is opposite to most of the proteins; therefore it may bind to other proteins by electrostatic interactions (Vachier *et al*., 1995). It can be expected that different populations of lysozyme molecules will be eluted with other proteins, resulting in lysozyme presence in peaks rather than in the first one, as described by Awadé and Efstathiou (1999).

It can be observed from the FPLC chromatogram (Fig. 4) that both chicken and tinamou egg white profile had a peak (A) which was reported to be lysozyme (Awadé and Efstathiou, 1999). FPLC fraction A was collected, concentrated and subjected to Bradford protein assay; no protein was present (Appendix A.1.1). When the concentrated sample was applied to SDS–PAGE no band was observed (Fig. 5). It is possible that this fraction contained only low molecular weight peptides. When the whole tinamou egg white was applied to SDS–PAGE, a band at 14 kDa, possibly attributable to lysozyme was present (Fig. 3). Also, when FPLC fractions B, C and D were applied to SDS–PAGE, a band of 12 kDa, possibly attributable to lysozyme, was observed. The identity of the band was confirmed by mass spectrometry as lysozyme C (as chicken lysozyme), even though lysozyme G is more widespread in other ratites species (Prager *et al.*, 1974).

These findings led us to examine the specific lysozyme enzymatic activity using the *M. lysodeikticus* assay. This assay is based on the observation that *M. lysodeikticus* cells are lysed when treated with

lysozyme. The rate of OD_{450} decrease due to cell lysis is directly proportional to the amount of lysozyme activity in the assay. FPLC Fraction A (lysozyme tentative fraction) had almost no activity (83 units/ml). One month old tinamou egg white samples had higher activity (1,700 units/ml), but only 10% of that present in "fresh" (4 days old) tinamou egg white (17,000 units/ml).

Goldberg *et al.* (1991) have found that lysozyme activity of chicken egg white is 34,200 unit/ml and that after reduction, exhibited almost no activity That may explain why the lysozyme activity of FPLC A fraction was very low. The FPLC fraction was pretreated with a reducing agent, β–mercaptoethanol, which can lead to the reduction of the lysozyme. It is not clear why the "old" tinamou egg white obtained a very low activity; more research is needed to understand the long term storing effects on tinamou lysozyme.

When the whole egg whites were applied to SDS–PAGE, there was a band matching the lysozyme molecular weight only for tinamou, and not for chicken and emu (Fig. 3). Several research groups have studies lysozyme content of emu egg white: Feeney *et al.* (1960) determined that the emu egg white contains of 0.05% lysozyme by *M. lysodeikticus* assay. Later on, Koga (1969) found the same data by a chromatographic separation of emu egg white on CM–cellulose and Takeuchi and Nagashima (2010) did not observe lysozyme on their SDS–PAGE. More recently, Maehashi *et al.* (2012) cloned and characterized emu lysozyme G and C genes. They concluded that emus have functional genes for both lysozyme G and C, similar to many other avian species, and the lysozyme G gene is expressed in oviduct probably as in other ratite; however, its expression levels in egg white were too low to be detected. It is possible that emu egg white expresses other antimicrobial proteins than lysozyme.

5.2.2 Ovotransferrin

Ovotransferrin, one of the major chicken egg white antimicrobial proteins, has a molecular weight of 76.6 kDa and an isoelectric point of 6.1 (Mine, 1995).

Ovotransferrin was the main protein of interest for this research; therefore it was more extensively studied. The SDS–PAGE profile (Fig. 3) of tinamou, chicken and emu egg whites analyzed by Image Quant TL software to determine the molecular weights of the different proteins revealed an 86 kDa band for both chicken and tinamou, and a 92 kDa band for emu. Though the molecular weights are higher than the previously determined molecular weight of chicken ovotransferrin, these proteins were identified as ovotransferrin, by comparing to the literature. Awadé and Efstathiou, (1999) determined that the second major FPLC peak is chicken ovotransferrin and Maehashi *et al*. (2010) determined that emu egg white SDS–PAGE band of a 78 kDa was ovotransferrin.

The SDS–PAGE profile (Fig. 3) was also analyzed by Image Quant TL software to determine the relative optical densities of each of the proteins bands. This analysis demonstrated that tinamou egg white contained approximately 30% more ovotransferrin than chicken. Emu egg white contained approximately 21% more ovotransferrin than tinamou and approximately 60% more than chicken.

Chicken ovotransferrin, was found to comprise 12% of the entire egg white, as described in several references. Feeney *et al*. (1960) determined by absorption of the ovotransferrin iron complex that emu egg white contains 10% ovotransferrin. Takeuchi and Nagashima (2010) compared chicken egg white and emu egg white on 12.5% Coomassie stained SDS–PAGE, and a thicker and darker band was observed for the emu ovotransferrin. Maehashi *et al*. (2010) did the same comparison with a 15% gel. They were able to detect a 78 kDa band, as the most abundant protein in emu egg white, comprised 33% of the total emu egg white proteins. It was identified as ovotransferrin due to its high N–terminal sequence similarity to that of chicken egg white. Additionally, they determined that emu ovotransferrin had the same molecular mass as chicken ovotransferrin.

It is possible that emu egg white has different protein distribution than chicken. Since emu ovotransferrin quantity appeared to be high, it would be interesting to further investigate it. Chilean tinamou egg white ovotransferrin quantity had not been determined before.

The observations that tinamou egg white contained more ovotransferrin than chicken egg white and that chicken and tinamou egg white contain similar amounts of iron, (0.59±0.16 and 0.43±0.02,

respectively) contradicted the second hypothesis. This hypothesis suggested that the fresh Chilean tinamou egg white is pink due to high quantities of iron saturated ovotransferrin. Since iron quantities of chicken and tinamou egg whites were similar, and ovotransferrin quantities were higher for tinamou, Chilean tinamou egg white does not contain a high quantity of iron-saturated ovotransferrin resulting in the pink hue. More research has to be conducted in order to determine why the fresh Chilean tinamou egg white is pink.

The next step for ovotransferrin characterization was separating chicken and tinamou egg white with anion–exchange FPLC. According to Fig. 4, it appeared that peak B, C, and D contained ovotransferrin. All fractions were subjected to deglycosylation, confirming the protein was glycosylated, similar to the chicken ovotransferrin. The deglycosylated band was outsourced for MS, confirming its identity as ovotransferrin. Peptide mass fingerprinting was performed by ESI MS/MS with a MASCOT database search for protein identification as chicken ovotransferrin, as seen on Fig. 7. Though mass spectrometry analysis confirmed ovotransferrin identify, disappointingly, only 9% of the total sequence corresponding to region with an unknown biological function was obtained. At the time this assay was conduct (2011) the emu ovotransferrin amino acid sequence was not available in the MASCOT database. By comparing the recently sequenced emu ovotransferrin (Maehashi *et al*., 2012) it can be observed that the tinamou ovotransferrin sequence obtained was highly homologous with emu ovotransferrin. A possible next step could be to obtain additional tinamou ovotransferrin amino acid sequence data, in order to determine which species the tinamou protein is more similar to. This could be done via cDNA analysis and translation. Knowledge of the tinamou ovotransferrin amino acid sequence will demonstrate whether there are differences between tinamou and chicken ovotransferrins, which can result in functionality differences; for example whether there are differences in the antimicrobial cationic regions in tinamou ovotransferrin compared to chicken ovotransferrin.

5.2.3 Ovalbumin

Ovalbumin is the most abundant protein in chicken egg white, comprising 54% of the total egg white proteins, and has a pI of 4.5 and a molecular weight of 45 kDa (Mine, 1995).

SDS–PAGE separation of the whole chicken, tinamou and emu egg whites, demonstrated that both chicken and tinamou, but not emu egg white, contain a band which can be associated with ovalbumin (Fig. 3). This difference suggests that the ovalbumin of emu egg white ovalbumin was either post–translationally modified or replaced by another protein with a similar biological function. For example, there was an unidentified 134 kDa high intensity band in the emu egg white protein SDS–PAGE profile that was not present in the chicken or tinamou. Maehashi *et al.* (2010) suggested that emu egg white contains a 102 kDa ovalbumin, determined by amino acid sequencing and a comparison to the chicken ovalbumin sequence. Chicken and tinamou egg white profiles contained bands at 30 kDa and 46 kDa for chicken, and 38 and 47 kDa for tinamou. The lower molecular weights can be attributed to ovalbumin. Even though the reported molecular weight of chicken ovalbumin is higher, the high intensity of these bands coincides with the prevalence of ovalbumin as the major egg white protein. The bands that were identified as 47 kDa and 46 kDa for chicken and tinamou, respectively, can be associated with ovoglobulin G2 and G3 (Stevens, 1991; Awadé and Efstathiou, 1999), which have a molecular weight of 47 kDa and 50 kDa, respectively. As previously determined that ovoglobulins comprise 2% of the total chicken egg white (Stevens, 1991), the ovoglobulin band had low intensity. SDS–PAGE analysis of FPLC fraction C–G of tinamou egg white revealed a 42 kDa band which can be associated with ovalbumin and an additional band of 51 kDa, identified as ovoglobulin. Since ovalbumin pI is 4.5 and ovoglobulin is 4.9, it is possible that those proteins would elute simultaneously.

Fractions C, D and E were further concentrated and treated with PNGase–F in order to deglycosylate the proteins. Untreated and treated fractions were subjected to SDS–PAGE, and the relative migration distance was measured. PNGase–F treatment caused a MW decrease of 7 kDa in the tinamou ovalbumin band, respectively, confirming that ovalbumin is an N–linked glycoprotein.

5.2.4 Ovomucoid

Ovomucoid is a glycoprotein of 28 kDa, comprises 11% of the chicken egg white proteins and has an isoelectric point of 4.1 (Li–Chan *et al.*, 1995).

SDS–PAGE separation (Fig. 3) of the whole chicken, tinamou and emu egg whites, demonstrated that only tinamou, contained a band which can be clearly associated with ovomucoid, with molecular weight of 23 kDa. Since it is well known that ovomucoid is one of the major chicken egg white proteins, this result is probably due to poor band protein separation (Koga, 1969). As for emu egg white ovomucoid, Feeney *et al*. (1960) studied on the avian egg whites from the standpoint of the comparative biochemical aspect and reported that the emu egg whites had high ovomucoid content with anti-proteolytic action strength and types different from those of the chicken egg white. Rhodes *et al.* (1958) and Koga (1969) found by chromatography on CM–cellulose column and electrophoresis that approximately 20% of the total proteins in the emu egg white were ovomucoid, which exceeded that of the chicken egg white. More recently, Maehashi *et al.* (2010) found that ovomucoid levels in emu egg white were estimated to be lower than those in chicken egg white by Western blotting, enzyme–linked immunosorbent assays using anti–chicken ovomucoid antibodies and SDS–PAGE. Our emu egg white SDS–PAGE profile did not contain ovomucoid band, probably due to poor separation of ovalbumin and ovomucoid (Koga, 1969). Additionally, the band which did associated with ovomucoid, as part of tinamou egg white proteins, was a low intensity band, despite the fact it was shown to be 11–20 % of some avian species (Feeney *et al.*, 1960). The putative tinamou ovomucoid band was further investigated by mass spectrometry, confirming its identity with a 5.9 kDa tinamou ovomucoid fragment. This fragment was determined previously by Apostol *et al.* (1993), as part of their investigation of a specific ovomucoid domain from 27 species.

5.2.5 Ovomucin and high molecular weight proteins

Ovomucin is a glycoprotein which consists of two subunits linked together by disulfide bonds; one subunit is carbohydrate–rich β–ovomucin with a molecular weight of 400–610 kDa and the second is

carbohydrate–poor α–ovomucin with a molecular mass of 254 kDa. Ovomucin comprises 3.5% of egg white proteins (Mine, 1995), and due to its high viscosity, it was removed from egg white prior to anion exchange analysis on the FPLC. There was a band of 189 kDa in the tinamou egg white profile, which may be as a residual amount of ovomucin. The whole tinamou and emu egg white contained a band of 173 kDa. It is not clear why those high molecular weight proteins were not present in the chicken egg white profile.

5.3 Determination of ovotransferrin antimicrobial activity

The second aim of the study was to determine the antimicrobial activity against pathogenic *E. coli* O157:H7 and *S. aureus* COL of chicken and tinamou ovotransferrin solutions.

As a preliminary step, the bacteriostatic activity of commercial chicken ovotransferrin was tested against non–pathogenic *E. coli* K-12 and *S. aureus*. Following that, bacteriostatic and bactericidal activity of native tinamou and chicken (1 and 10 mg/ml) ovotransferrins was determined against pathogenic *E. coli* O157:H7 and *S. aureus* COL. The last step was determining the bacteriostatic and bactericidal activity of apo and holo tinamou and chicken (5 and 10 mg/ml) ovotransferrins against *E. coli* O157:H7.

5.3.1 Determination of commercial chicken ovotransferrin bacteriostatic antimicrobial activity against *E. coli* K-12 and *S. aureus*

The bacteriostatic activity of commercial chicken ovotransferrin was tested against non–pathogenic *E. coli* K-12 and *S. aureus*, in order to obtain preliminary data on the bacteriostatic antimicrobial activity of ovotransferrin. Two concentrations were examined, 1 and 10 mg/ml, combined with three different concentrations of bicarbonate of 0, 50 and 100 mM (Fig. 20-23, Appendix A.2). Negative controls were samples containing distilled water instead of BHI medium. Positive controls were samples which contain distilled water instead of $NaHCO_3$ and ovotransferrin in BHI. The trend observed was that during the incubation period of 24 hr both *E. coli* K-12 and *S. aureus* grew significantly in the BHI sample, and in the positive control samples. There was no bacterial growth in

the negative control samples as expected. A concentration of 50 mM $NaHCO_3$ did not inhibit the growth of *E. coli* K-12 and *S. aureus*, though when combined with 1 or 10 mg/ml ovotransferrin, a significant inhibition was observed. However, a concentration of 100 mM $NaHCO_3$ was bacteriostatic towards both *E. coli* K-12 and *S. aureus* alone and when combined with 1 or 10 mg/ml ovotransferrin. Corral *et al.* (1988) determined that 120 mM $NaHCO_3$ reduced *E. coli* K-12 and *S. aureus* population by 4 log (CFU/ml). The bicarbonate ion was identified as the probable cause of inhibition in BHI broth and not changes in pH as was determined by examining samples with and without bicarbonate at different pH levels. From these observations, a concentration of 50 mM $NaHCO_3$ was selected for use in evaluation of the ovotransferrins against the two pathogens.

5.3.2 Determination of the antimicrobial activity of native, apo and holo chicken and tinamou ovotransferrins against *E. coli* O157:H7

Bacteriostatic and bactericidal activity of native, apo, and holo chicken and tinamou ovotransferrins against *E. coli* O157:H7 was determined by turbidity and viability assays, respectively.

Native ovotransferrins were tested at the following four conditions: 1 mg/ml and 10 mg/ml, with and without 50 mM $NaHCO_3$. Apo and holo ovotransferrins were tested at 5 mg/ml and 10 mg/ml, with and without 50 mM $NaHCO_3$.

Native chicken ovotransferrin was bacteriostatic against *E. coli* O157:H7 only at 10 mg/ml with 50 mM $NaHCO_3$, resulting in 95±1% inhibition (Fig. 8). However, tinamou ovotransferrin had a modest bacteriostatic effect against *E. coli* O157:H7 at both 1 and 10 mg/ml even without 50 mM $NaHCO_3$, with 8±2% and 16±2% inhibition, respectively. With 50 mM $NaHCO_3$, tinamou ovotransferrin at a concentration of 1 mg/ml did not have a bacteriostatic effect on *E. coli* O157:H7, while at 10 mg/ml, 96±1% inhibition was observed.

Interestingly, both chicken and tinamou ovotransferrins combined with 50 mM $NaHCO_3$, had the same bacteriostatic effect on *E. coli* O157:H7 for 1 and 10 mg/ml. At a concentration of 1 mg/ml with 50 mM $NaHCO_3$, both exhibited no bacteriostatic activity, whereas at a concentration of 10

mg/ml with 50 mM $NaHCO_3$ tinamou and chicken exhibited 96±1% and 95±1% inhibition, respectively.

According to the viability assay results (Table 5), both chicken and tinamou ovotransferrins at a concentration of 1 mg/ml with 50 mM $NaHCO_3$ reduced the CFU/ml by 1 log, whereas for the turbidity assay those samples had no inhibition at all. The reasons for this are not fully understood, and more research has to be done in order to determine chicken and tinamou ovotransferrin bacteriostatic activity against *E. coli* O157:H7 at lower concentrations of 1 mg/ml. It is important to mention that though turbidity assay is less time consuming and more cost efficient, it has some limitations. First, it does not distinguish between live and un-lysed-dead bacteria; therefore it is possible that a concentration of 1 mg/ml ovotransferrin with 50 mM $NaHCO_3$ did kill the bacteria, but cell lysis failed to occur, resulting in high absorbance values. A second limitation of turbidity assays is that even after the bacteria cells were lysed, it is possible that some of the bacteria content leaked to the media causing a false increase in absorbance. As a result of those limitations, a viability assay was conducted in order to determine ovotransferrin antimicrobial activity.

The results of the turbidity and viability assays indicated that both tinamou and chicken ovotransferrins are most effective against *E. coli* O157:H7 at a concentration of 10 mg/ml with 50 mM $NaHCO_3$. Under these conditions, both tinamou and chicken ovotransferrins significantly inhibited and moreover, killed the *E. coli* O157:H7 population.

It is not clear why tinamou ovotransferrin exhibited no bacteriostatic activity at a concentration of 1 mg/ml with 50 mM $NaHCO_3$, comparing to a minor activity of 8% at a concentration of 1 mg/ml alone; however, in general $NaHCO_3$ had a synergistic effect with both ovotransferrins that was most obvious at 10 mg/ml ovotransferrin. The antimicrobial activity of ovotransferrins and bicarbonate has been associated with ovotransferrins apo–forms, where it serves as a bridging ligand between ovotransferrins and iron, promoting iron sequestration (Aisen, 1980). Despite that, iron deprivation mechanism as the antimicrobial activity mode of ovotransferrin against *E. coli* strains has been controversial. Ibrahim *et al.* (1998 and 2000), determined that a 92 amino acid ovotransferrin peptide

had bactericidal activity against *E. coli* K–12, without NaHCO$_3$. However, Ko *et al.* (2008) demonstrated that apo–ovotransferrin combined with NaHCO$_3$ and EDTA had the potential to inhibit *E. coli* O157:H7 growth and that holo–ovotransferrin had little or no inhibitory activity against this strain. Additionally, it has been shown before that *E. coli* strains possess a variety of iron stress–response mechanisms and therefore may not be sensitive to iron deprivation state (Earhart, 1996; O'Brien and Gibson, 1970).

In order to further investigate the mechanism of ovotransferrin antimicrobial activity and the role of bicarbonate, a second experiment was executed, testing apo and holo ovotransferrins, with and without NaHCO$_3$.

The hypothesis was that if ovotransferrin is antimicrobial regardless of iron saturation state, both apo and holo ovotransferrins will exhibit antimicrobial activity against *E. coli* O157:H7, with or without NaHCO$_3$. However, if "iron–binding" is ovotransferrin antimicrobial mechanism, only apo–ovotransferrin 50 mM with NaHCO$_3$ will be antimicrobial.

Since the previous native ovotransferrins experiment resulted in dramatically different inhibition rates of ovotransferrin concentrations at 1 (no inhibition) and 10 mg/ml (95% inhibition), we decided to evaluate 5 and 10 mg/ml ovotransferrin in the next experiment.

The results demonstrated that there wasn't a significant difference between 5 and 10 mg/ml ovotransferrin, in all conditions (4–way ANOVA); this suggests that ovotransferrin antimicrobial activity may reach a level of saturation at 5 mg/ml.

In order to simplify the data analysis, a 3–way ANOVA (Appendix A.2.4) was constructed testing all the two level interactions between the significant main effects observed by the 4–way ANOVA. Since there wasn't any significant difference between 5 and 10 mg/ml ovotransferrins results, the following data analysis of the 3-way ANOVA discusses the specifics of 5 mg/ml ovotransferrins (Fig. 12). The results demonstrated that bacteriostatic activity against *E. coli* O157:H7 was bicarbonate dependent. In the absence of bicarbonate, apo ovotransferrins had a minor inhibition (7±4% and 2±4% for tinamou and chicken, respectively), while holo ovotransferrins exhibited no

inhibition at all against *E. coli* O157:H7. When bicarbonate was present, the overall inhibition was greater, and apo ovotransferrins inhibition was approximately 50% greater than holo ovotransferrins inhibition (Fig. 12). Both chicken and tinamou apo ovotransferrins had a significant bacteriostatic effect against *E. coli* O157:H7 of 58±8% and 50±5% inhibition, respectively. Both chicken and tinamou holo ovotransferrins had a lower bacteriostatic effect against *E. coli* O157:H7 of 25±8% and 15±4% inhibition, respectively. There was no interaction effect between ovotransferrin species and ovotransferrin type. Chicken apo and holo ovotransferrins had the same inhibition rates on average, as tinamou apo and holo ovotransferrin.

The viability assay results demonstrated that chicken and tinamou apo ovotransferrins at concentrations of 5 and 10 mg/ml with 50 mM $NaHCO_3$ reduced the bacteria population only by 1 log (CFU/ml). Similar results were obtained by Ko *et al*. (2009), which demonstrated that 20 mg/ml apo ovotransferrin with 50 mM $NaHCO_3$ reduced *E. coli* O157:H7 growth by 1 log (CFU/ml). Those results were different from our previous assay with native ovotransferrins, where ovotransferrins at 10 mg/ml with 50 mM $NaHCO_3$ reduced the bacteria population by at least 9 log (CFU/ml). Additionally, in the previous assay with native ovotransferrins this condition of 10 mg/ml with 50 mM $NaHCO_3$ had a dramatic bacteriostatic effect on *E. coli* O157:H7 as well, of ~95% inhibition, versus 50–58% obtained by apo ovotransferrins.

It is not clear why the antimicrobial activity of apo ovotransferrins differed so dramatically from native ovotransferrins. It is possible that due to the chemical manipulations executed in order to obtain apo and holo ovotransferrins, ovotransferrins were partially denatured and therefore their antimicrobial activity potential was decreased. It was determined before that ovotransferrin antimicrobial activity is affected by its structure, which could be altered by denaturation (Fraenkel–Conrat, 1950).

 Another speculation is that in the first experiment with native ovotransferrins, *E. coli* O157:H7 was in a different growth stage than in the last experiment with holo and apo forms. In the first experiment, *E. coli* O157:H7 was diluted 1000-fold with BHI, and immediately 5 µL were added to

each test well. In the last experiment, after 1000-fold dilution with BHI, one hour passed before adding 5 µL to each test well. In the first experiment *E. coli* O157:H7 was still in stationary phase, whereas in the last experiment *E. coli* O157:H7 was in exponential phase. It is expected that ovotransferrin inhibition would be greater in the exponential phase, although the opposite effect was observed. In the exponential phase, *E. coli* O157:H7 should have been more susceptible to ovotransferrin. In the stationary phase, *E. coli* O157:H7 was expected to be more resistant to the iron depleted conditions, since it produces iron-stress response factors, such as siderophores.

Those experiments should be repeated with bacteria in the exponential stage for more accurate results.

As found for native ovotransferrins, the antimicrobial activity against *E. coli* O157:H7 of chicken and tinamou native, apo and holo ovotransferrins was bicarbonate dependent. Upon bicarbonate presence, both chicken and tinamou ovotransferrins exhibited the same bacteriostatic and bacteriostatic characteristics against *E. coli* O157:H7.

In the absence of bicarbonate, tinamou ovotransferrins exhibited minor bacteriostatic activity, while chicken ovotransferrins had no bacteriostatic activity at all. It is possible that tinamou ovotransferrin possess different amino acid composition responsible for unique antibacterial motifs.

Additionally, this research demonstrated that besides the traditional hypothesis stating ovotransferrins are bacteriostatic against *E. coli* O157:H7 in their apo forms, holo ovotransferrins were found to be antimicrobial as well, albeit at a lower level. Since holo ovotransferrins antimicrobial activity was higher in the presence of bicarbonate, it is possible that bicarbonate contributes to the antimicrobial activity of ovotransferrin in a mechanism other than a bridging ligand between ovotransferrin and iron, such as membrane disruption.

In support of this hypothesis, Dorschner *el al*. (2006) showed that upon bicarbonate presence, cell wall structure and gene expression changed, whereas no change occurred in growth rate. Those gene and structure changes caused the bacteria to be more susceptible to antimicrobial peptides. For *E. coli,* a global alteration in gene expression was seen with more than 300 gene transcripts altered

greater than two-fold. Among the genes with significantly decreased expression was the global regulator *fli*A, which was confirmed by quantitative RT-PCR analysis.

Lastly, it can be inferred that ovotransferrin antimicrobial activity may reach a level of saturation at a concentration of 5 mg/ml, and therefore the antimicrobial activity mechanism might involve other mode of actions rather than iron binding. The iron content of the BHI media used was reported to be 0.44 µg/ml (Ahn *et al*., 2004). According to Valenti *et al*. (1982), ovotransferrin iron binding capacity is 1 µg iron per 40 mg ovotransferrin. A simple calculation shows that in order to bind all iron in the media the ovotransferrin concentration should be approximately 17 mg/ml. This suggests that removal of all available iron was not the reason for the same inhibition being exhibited by the 5 and 10 mg/ml ovotransferrin treatments. It would be interesting to determine the specific iron requirements of *E. coli* O157:H7, and by that determine whether the iron content in the media is sufficient for growth, despite ovotransferrin presence.

5.3.3 Determination of the antimicrobial activity of native chicken and tinamou ovotransferrins against *S. aureus* COL

Bacteriostatic and bactericidal activity of native chicken and tinamou ovotransferrins against *S. aureus* COL was determined by turbidity and viability assay, respectively, at 1 and 10 mg/ml ovotransferrin, with and without 50 mM $NaHCO_3$.

Addition of 1 and 10 mg/ml chicken ovotransferrin resulted in only 3% inhibition in the bacteriostatic assay while in the presence of bicarbonate, 61±3% and 68±5% inhibition was observed, respectively (Fig. 9). It is possible that chicken ovotransferrin effect on *S. aureus* COL reached saturation at 1 mg/ml, as the inhibition remain constant as protein concentration increased.

The bacteriostatic activity of tinamou ovotransferrin against *S. aureus* COL without 50 mM $NaHCO_3$ was 2±1% in samples containing either 1 or 10 mg/ml ovotransferrin. Higher inhibition of 15±2% was observed at 1 mg/ml with 50 mM $NaHCO_3$, and the highest inhibition rate of 95±1% was observed for tinamou ovotransferrin at 10 mg/ml with 50 mM $NaHCO_3$. Tinamou ovotransferrin

inhibition of *S. aureus* COL did not reach saturation at 1 mg/ml, as observed for chicken ovotransferrin, but was concentration dependent.

According to the viability assay results, no bacterial growth (e.g. at least a 9 log (CFU/ml) reduction) was observed on plates containing 10 mg/ml OTF with 50 mM NaHCO$_3$ for both chicken and tinamou, though according to the turbidity assay, the % inhibition of chicken ovotransferrin was significantly lower (61%) than the tinamou ovotransferrin (95%) under these conditions. The limitations of the turbidity assay were discussed previously; however, more research has to be done to determine chicken ovotransferrin bacteriostatic activity against *S. aureus* COL. Possible future directions of research are to include more technical and biological repeats of ovotransferrin samples and to examine intermediate native ovotransferrin concentrations such as 5 mg/ml. It is possible that the chicken ovotransferrin antimicrobial activity against *S. aureus* COL is more time–dependent than tinamou ovotransferrin; therefore future assays should involve longer incubation times and more frequent OD measurements.

Chicken ovotransferrin was more effective than tinamou ovotransferrin against *S. aureus* COL at 1 mg/ml OTF with 50 mM NaHCO$_3$. The bacteria population was reduced by 3 log (CFU/ml) in plates containing 1 mg/ml OTF with 50 mM NaHCO$_3$ chicken ovotransferrin, whereas tinamou ovotransferrin reduced the growth only by 1 log (CFU/ml). This coincides with the turbidity assay for this condition as the % inhibition of chicken ovotransferrin was significantly higher (68%) than that for tinamou ovotransferrin (15%).

Despite the similar effect between 1 and 10 mg/ml chicken OTF with 50 mM NaHCO$_3$ in the turbidity assay, but there was a large difference in the results of the viability assay (growth reduction by 3 logs vs. no growth). Again, the discrepancy between the viability and turbidity assays will require further investigation.

The viability assay results coincide with the turbidity assay results for samples containing 10 mg/ml ovotransferrin without 50 mM NaHCO$_3$, for both chicken and tinamou ovotransferrin. Both chicken

and tinamou at 1 and 10 mg/ml OTF without 50 mM $NaHCO_3$ did not have any bactericidal effect, and had little bacteriostatic effect on *S. aureus* COL.

Overall, observing the turbidity assay results, it seems tinamou ovotransferrin is more effective than chicken ovotransferrin against *S. aureus* COL, and it is concentration dependent. Only at a concentration of 10 mg/ml with 50 mM $NaHCO_3$ was there a significant inhibition of the growth of *S. aureus* COL. When a lower concentration of 1 mg/ml with 50 mM $NaHCO_3$ was applied, chicken ovotransferrin was more effective against *S. aureus* COL, though the % inhibition was moderate.

By observing the viability assay results, it seems tinamou and chicken ovotransferrin has similar concentration dependent bactericidal effects against *S. aureus* COL. Only at a concentration of 10 mg/ml with 50 mM $NaHCO_3$ was the viability of *S. aureus* COL significantly affected. When a lower concentration of 1 mg/ml with 50 mM $NaHCO_3$ was applied, chicken ovotransferrin was more effective against *S. aureus* COL.

Though there are uncertain issues from this study, it can be clearly inferred that $NaHCO_3$ has a synergistic effect with both ovotransferrins. When both chicken and tinamou ovotransferrins were combined with 50 mM $NaHCO_3$, a significant growth inhibition occurred, comparing to samples which did not include $NaHCO_3$. Accordingly, it can be concluded that bicarbonate is essential for the antimicrobial activity of ovotransferrins. It is possible that bicarbonate serves as a bridging ligand between ovotransferrins and iron (Aisen, 1980). Chicken ovotransferrin iron binding, which deprives bacteria of essential iron, requires one molecule of CO_2 as CO_3^{-2} or HCO_3^{-1} per atom of Fe^{3+} (Schalabach and Bates, 1975). However recently, Dorschner *el al*. (2006) showed that bicarbonate alone can change cell wall structure and gene expression, without any change in growth rate. Those gene and structure changes caused the bacteria to be more susceptible to antimicrobial peptides. Analysis of *S. aureus* demonstrated significant changes in genes involved in virulence, stress response, and cell wall maintenance. *S. aureus* global regulatory gene *sig*B was significantly suppressed when it was grown in the presence of bicarbonate. Despite that, Ibrahim *et al.,* (1998 and 2000) showed that a short ovotransferrin peptide can exhibited antimicrobial activity regardless of

bicarbonate presence. OTAP–92, a short cationic sequence located in the N–terminal domain of ovotransferrin, can directly interact with the outer membranes of microorganisms. This sequence showed high similarity to a peptide region in insect defensins, which are active against Gram positive and Gram negative bacteria by blocking voltage–dependent K^+ channels.

There are other studies suggesting some *S. aureus* strains are resistant to chicken ovotransferrin. Valenti *et al.* (1980) supported the bicarbonate–iron binding theory, though they reported that 50 strains of *S. aureus* were resistant to native ovotransferrin combined with 50 mM $NaHCO_3$. They argued that *S. aureus* has an efficient iron transport systems that can overcome iron deficient conditions. That was determined by comparing the bacterial growth of a media containing ovotransferrin with a media where iron was precipitated with chromium salts. Similar growth rate was observed for both medias, suggesting iron binding as a mode of antimicrobial action of ovotransferrin. It is possible that the different observations have occurred due to different non–pathogenic *S. aureus* strains, since *S. aureus* pathogenesis is significantly influenced by iron uptake and metabolism mechanisms.

There have been a few studies regarding human transferrin and iron binding as the antimicrobial activity mechanism against *S. aureus* (Lin *et al.*, 1994; Schade, 1963; Lindsay *et al.*, 1995). All determined that *S. aureus* can extract iron bound to human transferrin, which has a stronger affinity to iron than ovotransferrin. Additionally, Friedman *et al.* (2006) showed that *S. aureus* is able to alter its gene expression upon changes in iron sources. Further, they demonstrated that regulated overproduction of acidic end–products brought on by iron starvation decreases local pH resulting in the release of iron from the host iron–sequestering protein transferrin. Despite that, Kadurugamuwa *et al.* (1987) suggested that the iron requirement of *S. aureus* is relatively low.

Overall, it seems that more research has to be done in order to determine the iron metabolism of *S. aureus* in the presence of ovotransferrin, as a first step towards understanding whether the antimicrobial mechanism of ovotransferrin is related to its iron binding capacity. As a further step, it will be beneficial to test the antimicrobial activity of apo and holo ovotransferrins and bicarbonate

against *S. aureus* COL, under more specific iron conditions. It will be useful to evaluate ovotransferrins and *S. aureus* COL iron affinity by using differently labeled iron in the media and differently labeled iron bound to holo ovotransferrin.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Chilean tinamou (*Nothoprocta perdicaria*) egg white characterization and comparison to chicken (*Gallus gallus*) and emu (*Dromaius novaehollandiae*) egg whites, demonstrated that tinamou and chicken egg whites have more similarities than tinamou and emu egg whites, though phylogenetic analysis places tinamous within ratites including emu, which classifies remotely to chicken.

Egg white characterization demonstrated that tinamou and chicken, rather than tinamou and emu, share more similarities in protein, sialic acid, ash and iron content as well as in protein composition.

Ovotransferrin was the main protein of the research and was characterized both for biochemical and for antimicrobial properties. Proteomic analysis demonstrated that ovotransferrin was the common protein among all three species. Though all species contained ovotransferrins with similar molecular weights, ratite species were found to contain the highest concentrations. It is possible that ovotransferrin has an essential antimicrobial function as one of the major avian egg white proteins, and therefore its presence is conserved among distinct species.

Antimicrobial activity analysis of chicken and tinamou egg white ovotransferrins against two foodborne pathogens, *Escherichia coli* O157:H7 and *Staphylococcus aureus* COL, demonstrated that ovotransferrins antimicrobial activity is bicarbonate dependent and effective at concentrations of 5 mg/ml or higher.

More specifically, native ovotransferrins were the most effective, followed by apo and holo ovotransferrins. Holo ovotransferrins exhibited moderate antimicrobial activity, only in the presence of bicarbonate; therefore it is possible that bicarbonate contributes to the antimicrobial activity of ovotransferrin in a mechanism other than a bridging ligand between ovotransferrin and iron, such as membrane disruption caused by a protein conformational change. It is also possible that bicarbonate modified the bacteria stress related gene expression, increasing thier susceptibility to ovotransferrin. Since all ovotransferrin types exhibited different levels of antimicrobial activity, it seems that the antimicrobial activity of ovotransferrin is not due to only one mechanism, and it is more complex.

Both chicken and tinamou ovotransferrins had significant antimicrobial activity against *E. coli* O157:H7 and *S. aureus* in the presence of bicarbonate. In the absence of bicarbonate, tinamou ovotransferrins exhibited minor antimicrobial activity, comparing to chicken ovotransferrins that had no antimicrobial activity at all. It is possible that tinamou ovotransferrin may possess different amino acid composition responsible for unique antibacterial motifs. Those antimicrobial domains may be responsible for different antimicrobial mechanism, such as membrane disruption. The first direction for future research will be to obtain more data regarding tinamou ovotransferrin amino acid sequence. Though tinamou ovotransferrin identity was confirmed, only 9% of the total sequence was obtained and found to be homologous to the chicken sequence. Obtaining more data regarding tinamou ovotransferrin amino acid sequence will demonstrate whether there are differences between tinamou and chicken ovotransferrins, which can result in functional differences and may answer the question of why the tinamou egg white has a pink color that fades with time.

In conclusion, tinamou ovotransferrin combined with bicarbonate was found to be bactericidal against two foodborne pathogenic bacteria; therefore it should be further investigated as a natural antimicrobial agent for use in food matrices, such as produce and dairy products or on food preparation surfaces.

Additionally, since the characterization of the tinamou egg white proteins demonstrated that tinamou egg white share biochemical and antimicrobial similarities with chicken egg white, it may serve as an alternative source to chicken egg white proteins. The next step of the research will be to study other functional properties of tinamou egg white such as anti–adhesive, immune–modulatory, anticancer, antihypertensive and antioxidant.

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APPENDIX

A.1 Egg white characterization

A.1.1 Determining protein content of FPLC fractions by the Bradford protein assay

The protein content of FPLC fractions pre and post concentration was determined by Bradford assay.

A standard curve was prepared from triplicates of bovine serum albumin at different concentrations.
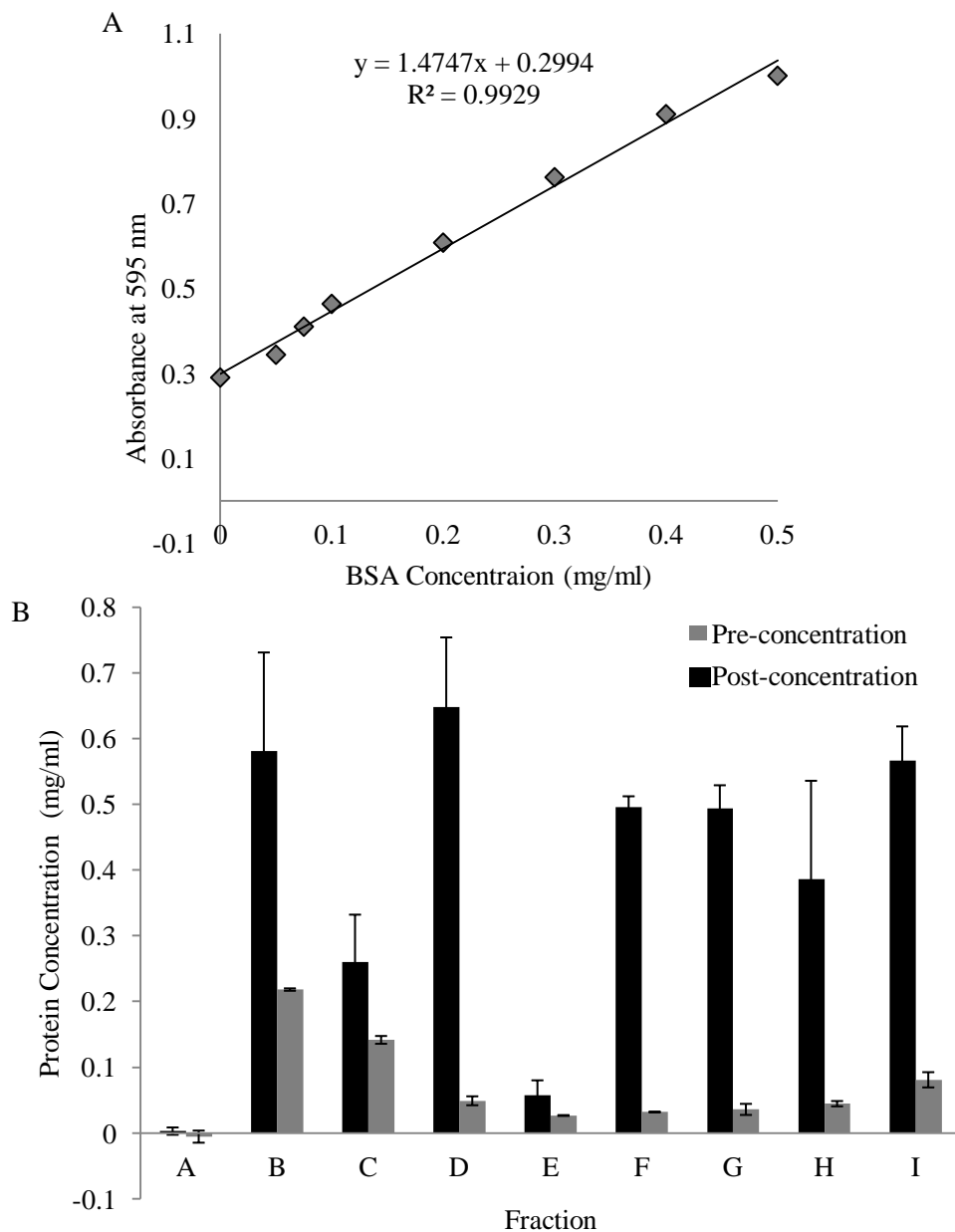

Figure 13 Bradford assay for tinamou egg white FPLC fractions. **A**) BSA (Bovine serum albumin) standard curve; **B**) FPLC fractions protein content before and after concentration;

A.1.2 Yield calculation of purified ovotransferrin production

Aliquots of 15 ml (collected in 15 runs) FPLC eluted "fraction B" (ovotransferrin fraction) were concentrated to give a 2 ml volume of concentrated protein solution and frieze dried to a fine powder. The following explains the yield calculation for purification of tinamou ovotransferrin:

Tinamou egg white contains approximately 15% ovotransferrin. Accordingly, we expected that in each 500 µl injected to FPLC column contains 0.75 mg ovotransferrin.

The theoretical calculation is as following:

- Proteins are 10% (100 mg/ml) of the entire egg white (according to Kjeldahl results)

- Ovotransferrin is approximately 15% (15 mg/ml) of the entire egg white proteins

- tinamou egg white is diluted x10 with sample buffer

→ tinamou egg white injection sample contains: 1.5 mg/ml OTF FPLC

- injection volume: 500 µl=0.5 ml

→ OTF content in the injection sample: 0.5 ml x 1.5 mg/ml OTF = 0.75 mg OTF

0.75 mg OTF X 15 injections = 11.25 mg OTF

→ According to Fig. 5, ovotransferrin was present on fractions C and D as well. According to a calculation of the relative optical density, only 60% of entire OTF was present in band B.

After 72 in the freeze drier, the concentrated OTF fraction 2ml gave 5.2 mg pure OTF (Fig. 14).

The relative practical yield is 100*5.2/0.6*11.25 = 77 %
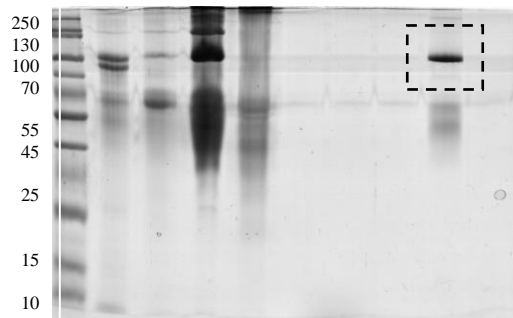

Figure 14 12.5% SDS–PAGE profile of purified 2 mg protein/ml tinamou ovotransferrin (in square).

A.1.3 Ferrozine assay standard curve

A standard curve was prepared from an iron standard solution using triplicates.
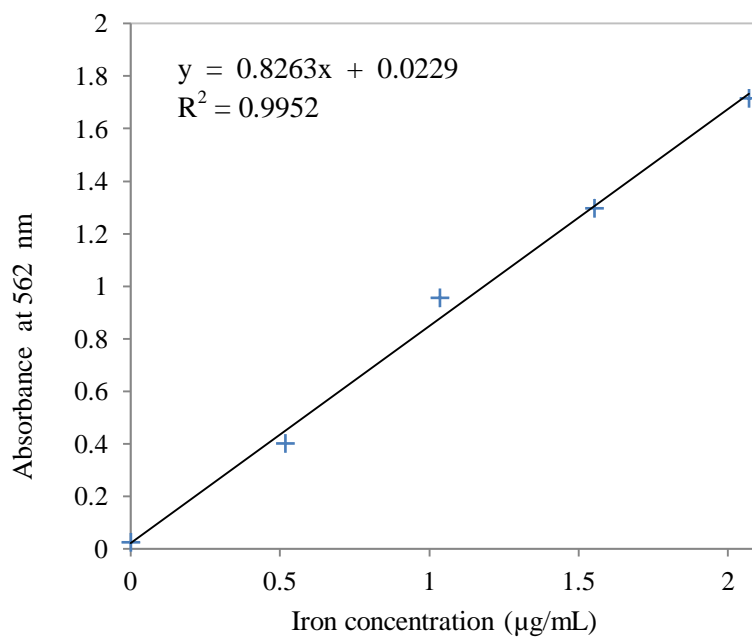

$$y = 0.8263x + 0.0229$$
$$R^2 = 0.9952$$

Figure 15 Standard curve for the ferrozine assay.

A.1.4 Sialic acid assay standard curve

A standard curve was prepared from triplicates of N–acetylneuraminic acid standard.

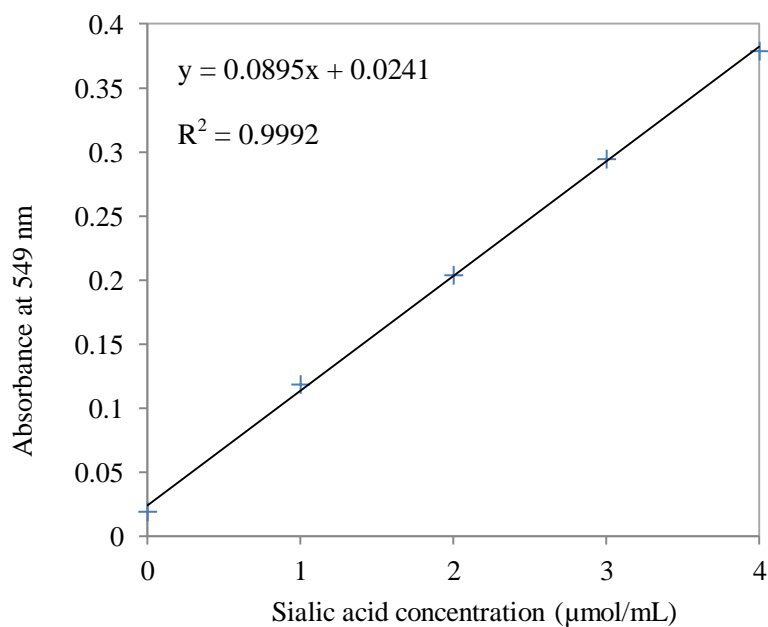$$y = 0.0895x + 0.0241$$
$$R^2 = 0.9992$$

Figure 16 Standard curve for the sialic acid assay

A.1.5 Lysozyme activity assay

The following graph represents the lysozyme activity assay results. The data presented is an average of three independent measurements.
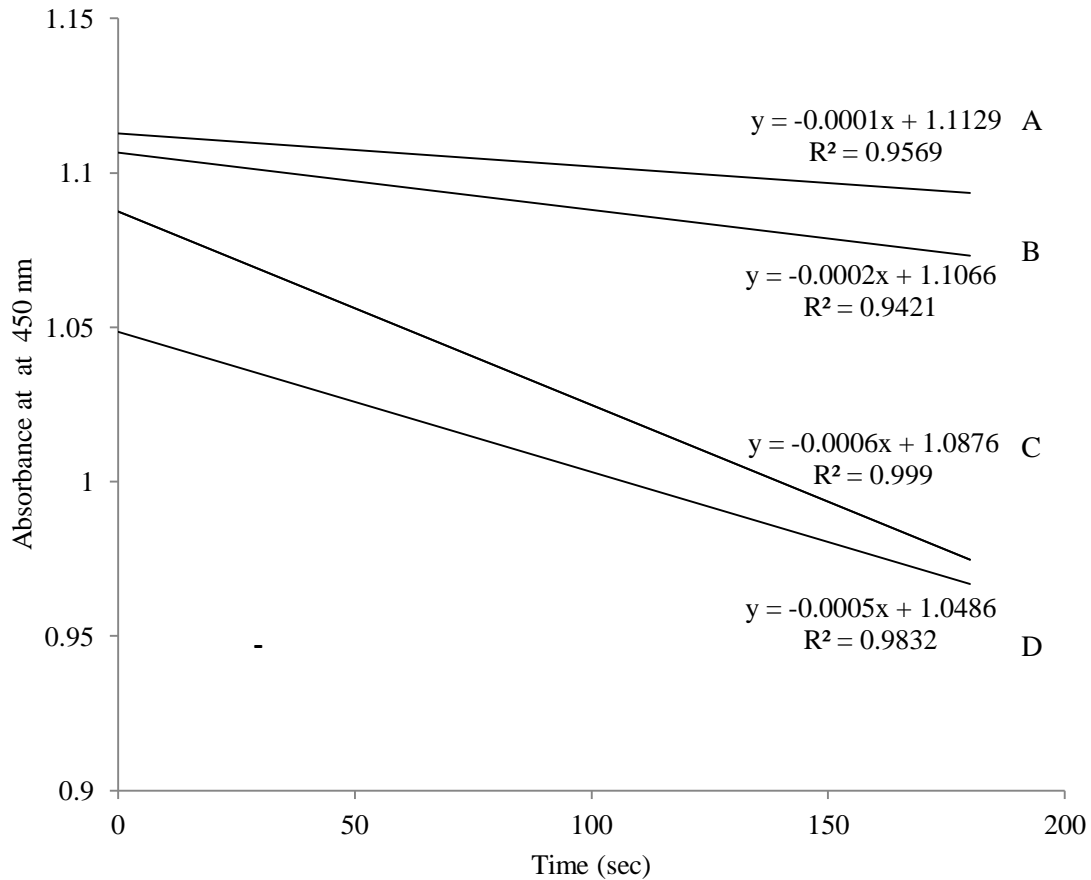

Figure 17 Determining lysozyme activity against *Micrococcus lysodeikticus* A) tinamou FPLC concentrated lysozyme fraction; B) "OLD" (1 month old) tinamou egg white; C) 500 units/ml chicken lysozyme (Sigma); D) "FRESH" (4 days old) tinamou egg white; Negative control=DW.

Calculation steps for determining tinamou egg white lysozyme activity

1. If pure chicken egg white lysozyme at 500 units/ml caused a OD_{450}/min=0.036, Then an OD_{450}/min=0.03, is 416.66 units/ml

2. Fresh tinamou egg white sample was diluted x40 ➔ 40*416.66 units/ml=16,666.67 $= 17*10^3$ units/ml in fresh tinamou egg white.

A.1.6 Determination of the relative ovotransferrin content on SDS–PAGE band

The comparison between ovotransferrin contents of egg whites was executed using the Image Quant TL software to analyze SDS–PAGE band intensities. The software measured the band optical density, which was proportional to the protein concentration.
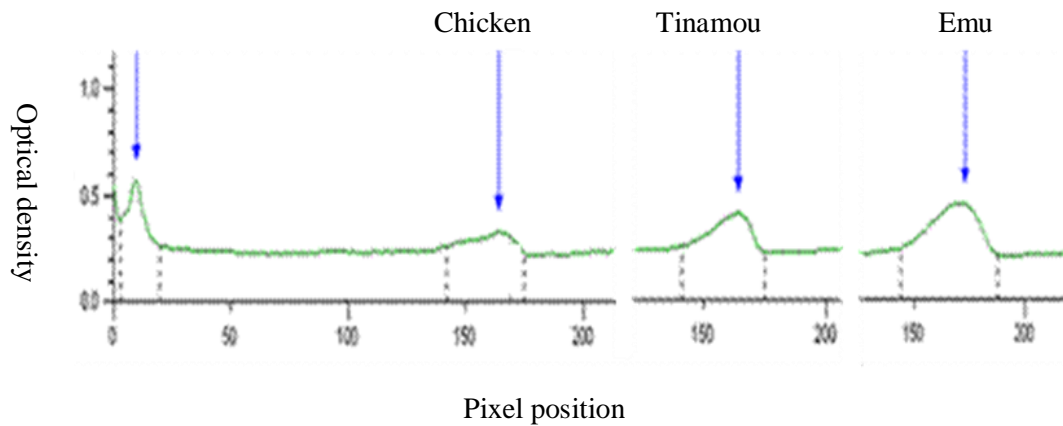

Figure 18 Determining the relative ovotransferrin content by the optical densities. Arrows point to the ovotransferrin band optical density of each species.

A.1.7 Deglycosylation of concentrated FPLC fractions by Peptide:N–glycosidase F (PNGase–F)

Glycosylation content of the ovotransferrin and ovalbumin was measured by the relative migration distance of the proteins band on the SDS–PAGE before and after PNGase–F treatment. The relative mobility (Rf) was calculated as the ratio of the distance migrated by the molecule to that migrated by a molecular weight marker logarithmic standard curve:
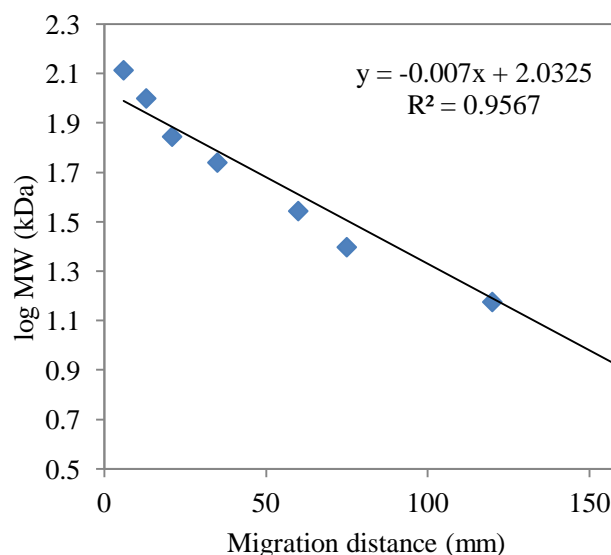
Figure 19 Molecular weight marker (12–230 KDa) migration distance standard curve on 12.5 % SDS–PAGE

The relative mobility data is presented on the next table:

Table 10 Relative mobility of ovotransferrin and ovalbumin SDS–PAGE bands with and without PNGase–F treatment

		Band location	log(MW)	MW	% Glycosylation
ovotransferrin	– PNGase	23	1.87	74	6
	+ PNGase	27	1.84	69	
Ovalbumin	– PNGase	54	1.65	45	15
	+ PNGase	64	1.58	38	

A.1.8 Trypsin digestion maps for chicken ovotransferrin, lysozyme and ovomucoid

Ovomucoid

```
1    MAMAGVFVLF  SFVLCGFLPD  AAFGAEVDCS  RFPNATDKEG  KDVLVCNKDL  RPICGTDGVT  YTNDCLLCAY  SIEFGTNISK
81   EHDGECKETV  PMNCSSYANT  TSEDGKVMVL  CNRAFNPVCG  TDGVTYDNEC  LLCAHKVEQG  ASVDKRHDGG  CRKELAAVSV
161  DCSEYPKPDC  TAEDRPLCGS  DNKTYGNKCN  FCNAVVESNG  TLTLSHFGKC
```

Lysozyme

```
1    MRSLLILVLC  FLPLAALGKV  FGRCELAAAM  KRHGLDNYRG  YSLGNWVCAA  KFESNFNTQA  TNRNTDGSTD  YGILQINSRW
81   WCNDGRTPGS  RNLCNIPCSA  LLSSDITASV  NCAKKIVSDG  NGMNAWVAWR  NRCKGTDVQA  WIRGCRL
```

Ovotransferrin

```
1    MKLILCTVLS  LGIAAVCFAA  PPKSVIRWCT  ISSPEEKKCN  NLRDLTQQER  ISLTCVQKAT  YLDCIKAIAN  NEADAISLDG
81   GQAFEAGLAP  YKLKPIAAEV  YEHTEGSTTS  YYAVAVVKKG  TEFTVNDLQG  KTSCHTGLGR  SAGWNIPIGT  LLHRGAIEWE
161  GIESGSVEQA  VAKFFSASCV  PGATIEQKLC  RQCKGDPKTK  CARNAPYSGY  SGAFHCLKDG  KGDVAFVKHT  TVNENAPDQK
241  DEYELLCLDG  SRQPVDNYKT  CNWARVAAHA  VVARDDNKVE  DIWSFLSKAQ  SDFGVDTKSD  FHLFGPPGKK  DPVLKDLLFK
321  DSAIMLKRVP  SLMDSQLYLG  FEYYSAIQSM  RKDQLTPSPR  ENRIQWCAVG  KDEKSKCDRW  SVVSNGDVEC  TVVDETKDCI
401  IKIMKGEADA  VALDGGLVYT  AGVCGLVPVM  AERYDDESQC  SKTDERPASY  FAVAVARKDS  NVNWNNLKGK  KSCHTAVGRT
481  AGWVIPMGLI  HNRTGTCNFD  EYFSEGCAPG  SPPNSRLCQL  CQGSGGIPPE  KCVASSHEKY  FGYTGALRCL  VEKGDVAFIQ
561  HSTVEENTGG  KNKADWAKNL  QMDDFELLCT  DGRRANVMDY  RECNLAEVPT  HAVVVRPEKA  NKIRDLLERQ  EKRFGVNGSE

641  KSKFMMFESQ  NKDLLFKDLT  KCLFKVREGT  TYKEFLGDKF  YTVISSLKTC  NPSDILQMCS  FLEGK
```

A.2 Determination of ovotransferrin antimicrobial activity

A.2.1 Determination of the bacteriostatic activity of commercial chicken ovotransferrin against *E. coli* K-12 and *S. aureus*

The bacteriostatic activity of commercial chicken ovotransferrin was tested against non–pathogenic *E. coli* K-12 and *S. aureus* as an initial step in order to obtain qualitative data. Two commercial chicken ovotransferrin concentrations were examined, 1 and 10 mg/ml combined with three different concentrations of bicarbonate: 0, 50 and 100 mM $NaHCO_3$. Fig. 20-23 represent the raw data that was collected for those assays.

Figure 20 The bacteriostatic activity of 1 mg/ml commercial chicken ovotransferrin against *E. coli* K-12 combined with three different concentrations: 0, 50 or 100 mM NaHCO₃. A control sample was examined for each sample. For sample "BHI", the control was a sample containing deionized water only. For all other samples, the controls contained deionized water instead of NaHCO₃ and ovotransferrin (+BHI).
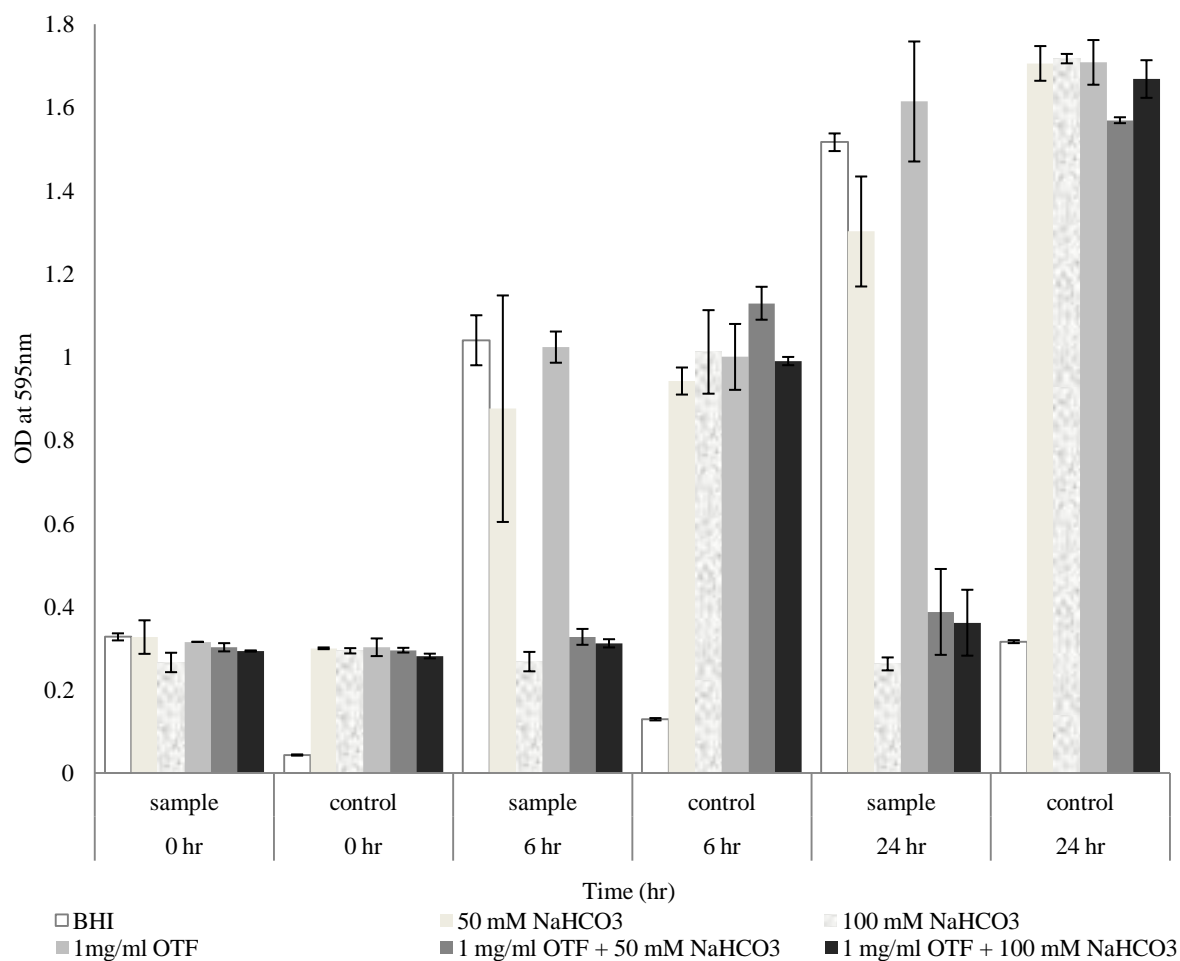
Figure 21 The bacteriostatic activity of 10 mg/ml commercial chicken ovotransferrin against *E. coli* K-12 combined with three different concentrations: 0, 50 or 100 mM NaHCO₃. A control sample was examined for each sample. For sample "BHI", the control was a sample containing deionized water only. For the rest of the samples, the controls contained deionized water instead of NaHCO₃ and ovotransferrin (+BHI).
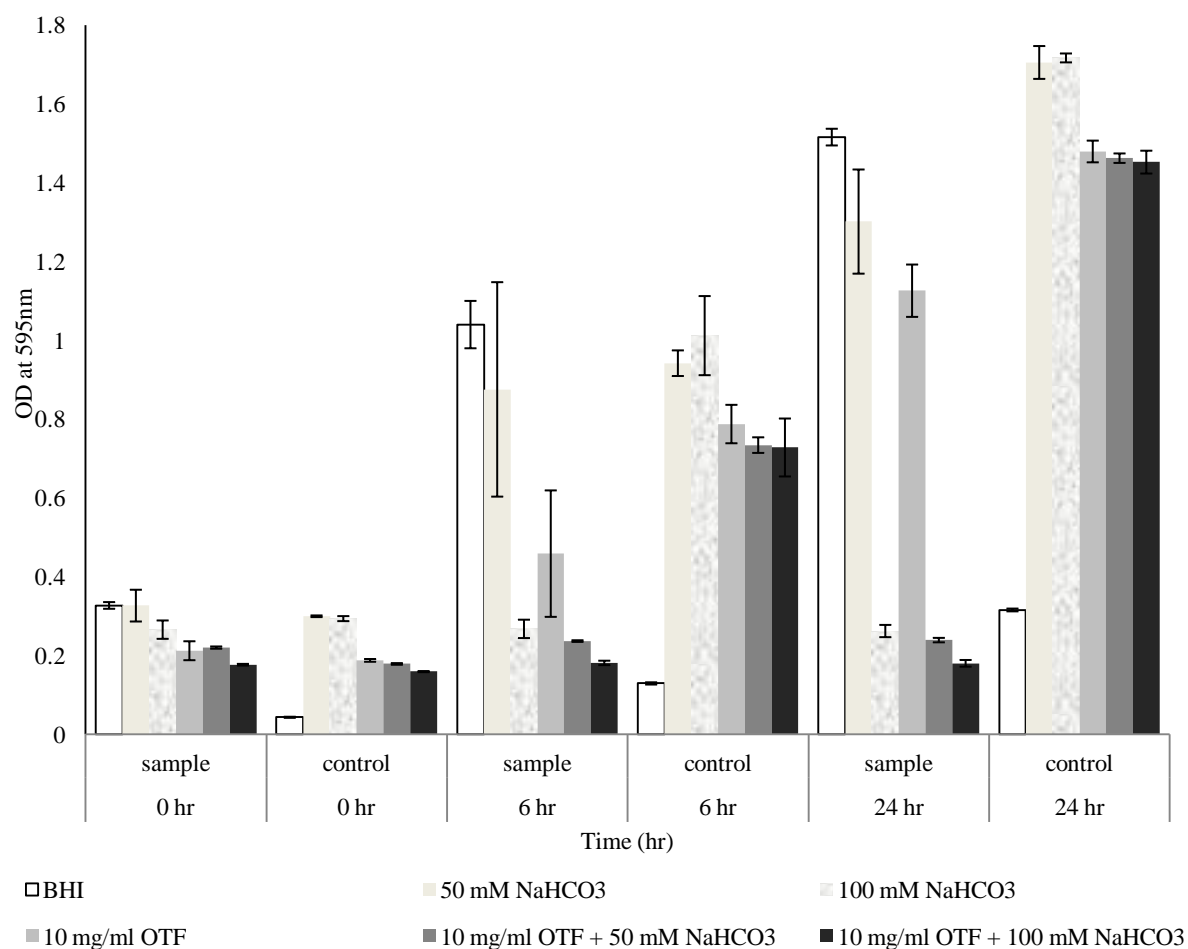
Figure 22 The bacteriostatic activity of 1 mg/ml commercial chicken ovotransferrin against *S. aureus* combined with three different concentrations: 0, 50 or 100 mM NaHCO₃. Test samples are highlighted in yellow. A control sample was examined for each sample. For sample "BHI", the control was a sample containing deionized water only. For the rest of the samples, the controls contained deionized water instead of NaHCO₃ and ovotransferrin (+BHI).
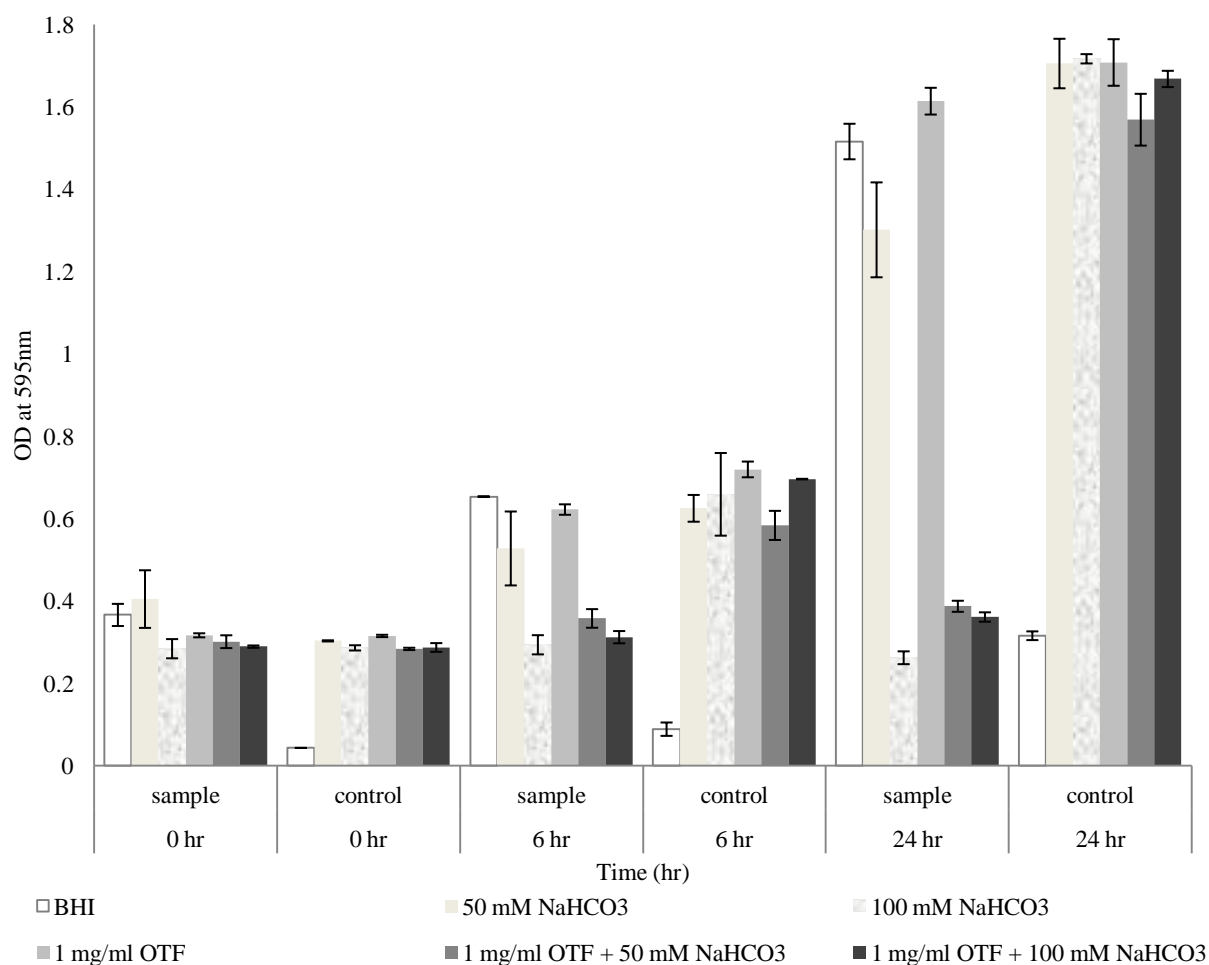
Figure 23 The bacteriostatic activity of 10 mg/ml commercial chicken ovotransferrin against *S. aureus* combined with three different concentrations: 0, 50 or 100 mM NaHCO$_3$.

A control sample was examined for each sample. For sample "BHI", the control was a sample containing deionized water only. For the rest of the samples, the controls contained deionized water instead of NaHCO$_3$ and ovotransferrin (+BHI).
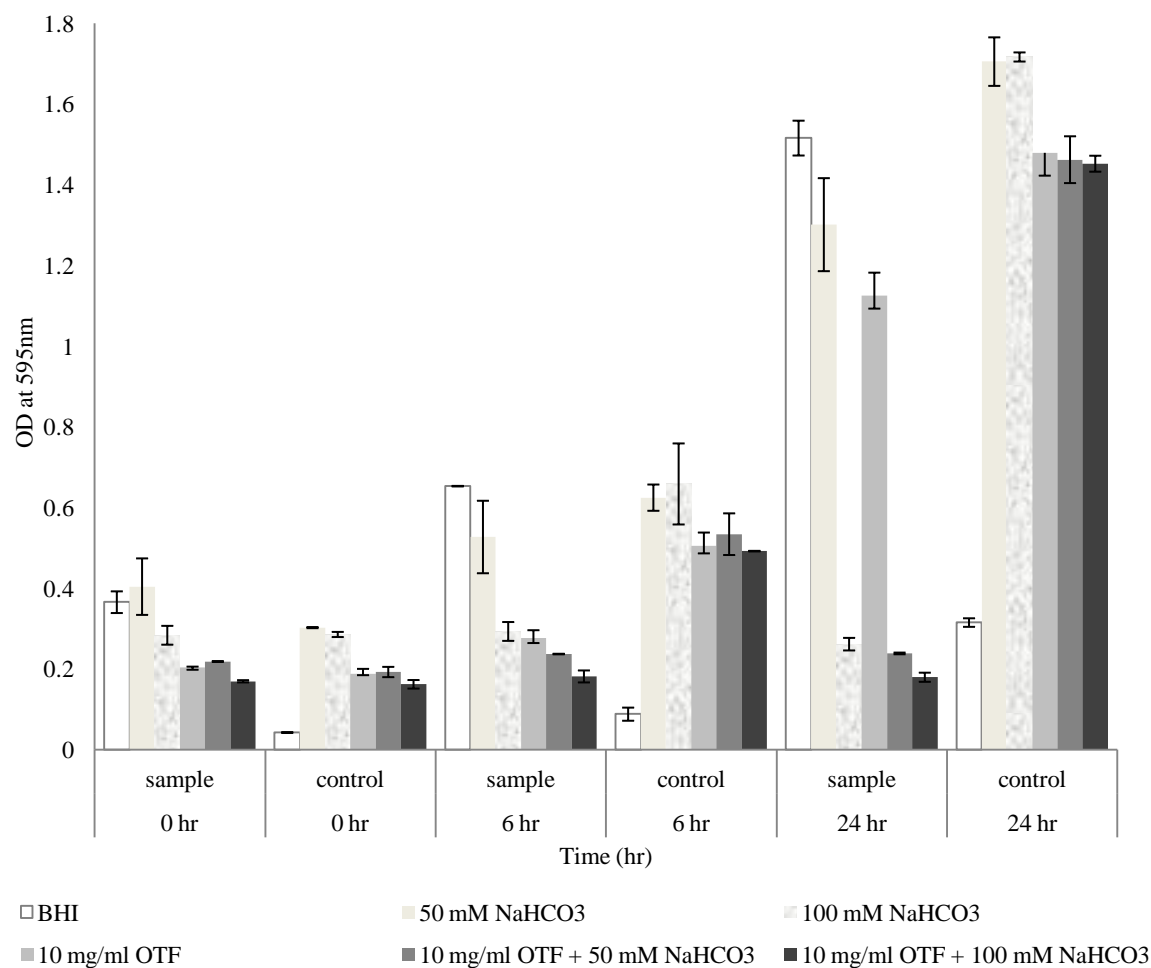
A.2.2 Apo and holo ovotransferrins concentration calculation

Calculation according to Fig. 10:

$A = \varepsilon cl$

$$A = \frac{4360(Mcm)^{-1} \times x\,^{mg}/_{ml} \times 1cm}{76600\,^{gr}/_{mol}} = 0.32(CH) \text{ or } 0.28(TH)$$

Chicken holo=CH=5.6 mg/ml

Tinamou holo=TH=4.9 mg/ml

A.2.3 Determination of the viability assay results

Colonies counted to determine the microbial population in viability assays, where calculate as follows:

Colonies forming Unit/milliliter (CFU/ml) =
$$\frac{A+B}{2} \times \frac{1}{\text{Subsequent dilutions}} \times \frac{1}{\text{Volume Plated}}$$

Notes:
− A and B are the duplicate plate counts; this is the average of the duplicate counts
− Subsequent dilutions:
  e.g. for dilution scheme going to 10^{-5}
   $10^{-1}= 0.1; 10^{-2}= 0.1; 10^{-3}=0.1; 10^{-4}=0.1; 10^{-5}=0.1$
    − i.e. 1/(0.1 x 0.1 x 0.1 x 0.1 x 0.1)
− Volume plated
  − spread plate = 100 ul= 0.1 ml (i.e. 1/0.1 ml)

A.2.4 Statistical analysis for turbidity assays for apo and holo ovotransferrins

Four–way ANOVA: Species, Iron, OTF conc, NaHCO$_3$

```
Source                          DF      SS      MS       F      P
Species                          1  0.07751  0.07751    4.69  0.033
Iron                             1  1.84365  1.84365  111.62  0.000
OTF conc                         1  0.04174  0.04174    2.53  0.116
NaHCO3                           1  2.10292  2.10292  127.32  0.000
Species*Iron                     1  0.00876  0.00876    0.53  0.469
Species*OTF conc                 1  0.19176  0.19176   11.61  0.001
Species*NaHCO3                   1  0.14344  0.14344    8.68  0.004
Iron*OTF conc                    1  0.14498  0.14498    8.78  0.004
Iron*NaHCO3                      1  0.36579  0.36579   22.15  0.000
OTF conc*NaHCO3                  1  0.08195  0.08195    4.96  0.029
Species*Iron*OTF conc            1  0.03478  0.03478    2.11  0.151
Species*Iron*NaHCO3              1  0.30909  0.30909   18.71  0.000
Species*OTF conc*NaHCO3          1  0.00648  0.00648    0.39  0.533
Iron*OTF conc*NaHCO3             1  0.00782  0.00782    0.47  0.493
Species*Iron*OTF conc*NaHCO3     1  0.27989  0.27989   16.95  0.000
Error                           80  1.32135  0.01652
Total                           95  6.96191
```

Three–way ANOVA: Species, Iron and Bicarbonate (5 and 10 mg/ml treated as repeats):

```
Source          DF  Seq SS   Adj SS   Adj MS     F      P
species          1  0.07751  0.07751  0.07751   2.85  0.095
iron             1  1.84365  1.84365  1.84365  67.81  0.000
bicarb           1  2.10292  2.10292  2.10292  77.34  0.000
species*iron     1  0.00876  0.00876  0.00876   0.32  0.572
iron*bicarb      1  0.36579  0.36579  0.36579  13.45  0.000
species*bicarb   1  0.14344  0.14344  0.14344   5.28  0.024
Error           89  2.41983  2.41983  0.02719
Total           95  6.96191
```

Three–way ANOVA: Species, Iron and Bicarbonate (5 mg/ml ovotransferrin only)

```
Source          DF  Seq SS   Adj SS   Adj MS      F      P
species          1  0.01272  0.01272  0.01272    4.15  0.048
iron             1  0.47732  0.47732  0.47732  155.57  0.000
bicarb           1  1.50757  1.50757  1.50757  491.36  0.000
species*iron     1  0.00431  0.00431  0.00431    1.41  0.243
species*bicarb   1  0.04448  0.04448  0.04448   14.50  0.000
iron*bicarb      1  0.24028  0.24028  0.24028   78.31  0.000
Error           41  0.12579  0.12579  0.00307
Total           47  2.41247
```

Three–way ANOVA: Species, Iron and Bicarbonate (10 mg/ml ovotransferrin only)

```
Source          DF  Seq SS   Adj SS   Adj MS     F      P
species          1  0.25656  0.25656  0.25656   5.89  0.020
iron             1  1.51131  1.51131  1.51131  34.72  0.000
bicarb           1  0.67730  0.67730  0.67730  15.56  0.000
species*iron     1  0.03923  0.03923  0.03923   0.90  0.348
species*bicarb   1  0.10544  0.10544  0.10544   2.42  0.127
iron*bicarb      1  0.13333  0.13333  0.13333   3.06  0.088
Error           41  1.78454  1.78454  0.04353
Total           47  4.50771
```