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Antibacterial activity of egg white: influence of physico-chemical conditions

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The egg white plays a major role in the protection of eggs against microorganisms. In addition to the shell barrier, it contributes to preserve the highly nutritive egg yolk from microbial invasion. In spite of such a protective system, eggs and egg contents may be contaminated by foodborne bacteria, which are real threats for the human health. In this context, understanding the antibacterial activity of egg white and the factors involved in its modulation is essential for a better control of the hygienic quality of eggs.

The egg white is a complex biological fluid that represents a harsh milieu for the growth of many bacteria. It contains an arsenal of natural antimicrobial molecules and mainly antibacterial proteins, including the well-known ovotransferrin and lysozyme, which exert their activities against a large panel of bacteria. In addition, a number of minor components with putative antimicrobial properties have recently been identified and/or characterized. Several physicochemical parameters, such as the natural alkaline pH, the ambient temperature, and the ionic environment might also modulate the activity of egg white antimicrobial components and/or increase the overall stress level impacting bacterial physiology. In egg white, the antimicrobial proteins could act in synergy with other proteins or with other egg white components. These activities could also be modulated by several physicochemical parameters, depending on the egg storage conditions.

The aim of the present review is to give a comprehensive overview of the key parameters controlling the bacterial growth in egg white. The actual knowledge on antimicrobial molecules, their mode of action, the bacterial resistance, and the involvement of physicochemical conditions on their activity will be described. Potential applications will be highlighted on egg storage practices, in a view to maintain or increase egg white antimicrobial activities during egg storage.

Keywords: egg white; antibacterial molecules; alkaline pH; temperature; physicochemical conditions.

Introduction

The avian egg white (albumen) is an aqueous solution of proteins. It is composed of 84-89% of water, the dry matter being constituted mostly of proteins (90%) and few amounts of carbohydrates, vitamins and minerals. The major function of these constituents is to provide nutrients and to protect the developing embryo and the highly nutritive egg yolk against invading bacteria. Egg white is a biological fluid secreted into the oviduct lumen by tubular glands and epithelial cells of the magnum, a specific segment of the oviduct. The egg white surrounds the egg yolk before being covered by eggshell membranes and the egg shell. Egg white is poor in nutrients available for the microorganisms (e.g. iron) and contains a great number of molecules that contribute to its remarkable antimicrobial properties. It is well established that avian egg white possesses antibacterial properties. The first demonstration of bactericidal effect was probably done by Wurtz in 1890 on *Bacillus anthracis*. These findings were confirmed later in 1909 by Laschtschenko. A thermolabile enzyme was suspected to be involved in this activity. This bactericidal molecule was firstly identified by Fleming in 1922 and named lysozyme. Thereafter, additional egg white proteins with antibacterial properties were reported.

In addition to the shell and eggshell membranes, the egg white participates in the natural egg defences by preserving the developing embryo in hatching eggs from bacterial contaminations and by ensuring the hygienic quality of table eggs. In spite of such efficient system, eggs and egg contents can be contaminated by a variety of bacterial species including foodborne bacteria. The most well-known, *Salmonella enterica* Enteritidis is the predominant serotype being responsible for 90% of foodborne diseases (salmonellosis) due to egg or egg product consumption (EFSA, 2009). The consumption of raw or undercooked eggs constitutes the main risk factor. The hygienic quality of eggs is still not completely under control despite many efforts from authorities, the poultry and egg industry, researchers and the pharmaceutical and feed industry. The risk of contamination by *Salmonella* is a real threat for human health and remains a major concern for the egg producing and the egg product manufacturing industry. Under healthy breeding conditions, the egg content is generally sterile just after laying. However, it can be contaminated by a diversified microbiota containing food spoilage microorganisms and sometimes pathogenic bacteria. Eggs can be contaminated externally, on the egg shell, and internally, i.e. during its formation (See Baron and Jan, 2011, for review). In the case of *Salmonella*, eggs may be contaminated during their formation in the ovary or in the oviduct of infected hens (vertical contamination). However, this type of contamination is sporadic. The exogenic contamination of eggs is by far more frequent than the vertical contamination for overall bacteria and corresponds to the contamination of the eggshell surface and subsequent penetration of microbes into the egg. It occurs after laying, when the eggshells are in contact with hen's faecal microorganisms or with other microorganisms present in the farm environment, or, downstream, in the environment of the conditioning centres. Contamination may also occur during egg production or transport and/or packaging in farm or in the conditioning centre, either by the environment, or from one egg to another. The presence of cracked eggs increases the risk of internal contamination.

The present review aims to give a comprehensive overview of the key parameters controlling the bacterial growth in egg white. In the first part, a repertoire of major and minor egg white proteins with antibacterial activities will be described with particular emphasis on their mode of action and their relative importance. The second part will discuss the physicochemical and environmental conditions (temperature, pH, viscosity, carbon dioxide, ions) influencing the bacterial growth or the activity of antibacterial proteins. The last part of this review explores the modifications of the egg white, its physicochemical and antibacterial properties observed during egg storage and highlights potential applications and innovative methods related to egg preservation to maintain or increase egg white antimicrobial activities.

I. Egg white antimicrobial molecules

A number of macromolecules, such as lysozyme which lyses Gram-positive bacteria, ovotransferrin which deprives bacteria of iron, and proteinase inhibitors have all been commonly cited to explain the ability of egg white to prevent bacterial growth. These molecules are abundant in the egg white, but it is noteworthy that some minor components recently revealed by high throughput approaches might play also a role in the defense against bacterial contaminations. The effect of most of these compounds is mediated according to three mechanisms: direct lysis of the microorganism, chelation of essential nutrients and/or inhibition of microbial enzymes.

I.1. Lysozyme

Lysozyme, present in many biological fluids, is an important component of the non-specific defence mechanisms. It is responsible for different types of bactericidal activity. Lysozyme is a basic protein (isoelectric point is 10.7) of 14307 Daltons (129 amino acid residues), a molecular weight which is low compared with most proteins and enzymes. The structure of lysozyme consists of two domains in which one is predominantly composed of helical structures whereas the other domain contains β -sheets. Its amino-acid sequence is cross-linked by four disulfide bridges. The enzymatic activity of lysozyme is lost when more than two disulfide bridges are destroyed.

One of the major antimicrobial mechanisms of lysozyme, and the best known, is the degradation of the glycosidic (1-4) β -linkage between the N-acetylglucosamine and the N-acetylmuramic acid of the peptidoglycan layer in the bacterial cell walls (muramidase activity). The peptidoglycan is a strong,

woven mesh that maintains the cell's shape and allows the diffusion of solutes and other type of molecules via large openings in the mesh. The cell wall determines the shape of the cell and protects cells from osmotic lysis. Without a wall or when it is attacked by lysozyme or other antimicrobial agents, the cell would swell and burst. Bacteria that are susceptible to the enzymatic action of lysozyme are not lysed when they are in an osmotically balanced medium. Cell death occurs by the lytic action of lysozyme on peptidoglycan only when in low-osmotic-strength media, or when the rate of the synthesis and polymerization process for new peptidoglycan formation is slower than the lysozyme catalyzed degradation.

Such a lysozyme mechanism is unfortunately, limited to certain Gram-positive bacteria. The peptidoglycan of some Gram-positive bacteria is indeed resistant to hydrolysis by lysozyme because of chemical modifications. This is the case for *Staphylococcus aureus* which carries an O-acetylation on the C-6 hydroxyl group of the N-acetylmuramyl residues (Clarke and Dupont, 1992), and for *Bacillus subtilis*, which has a de-N-acetylation on the N-acetylglucosamine group (Atrih et al., 1999). Peptidoglycan of pathogenic species is often modified, allowing them to resist to host defenses (Vollmer, 2008). Lysozyme is very basic and therefore positively charged at physiological conditions and can bind to more or less abundant negatively charged teichoic acids depending on the species, preventing access to its peptidoglycan substrate.

Invasive Gram-negative bacteria are resistant to the lytic action of lysozyme. The cell walls of Gram-negative bacteria have a more complicated structure, which is constituted by a thin layer of peptidoglycan surrounded by the outer membrane (lipid membrane containing lipopolysaccharide and lipoproteins). The peptidoglycan layer in Gram-negative bacteria is protected from the lytic action of lysozyme because the outermost surface of the bacteria functions as a permeability barrier. Lysozyme is trapped and inactivated by surface lipopolysaccharide (LPS) of the outer membrane of these bacteria (Ohno and Morrison, 1989). More broadly, in addition to the protection provided by their outer membrane, Gram-negative bacteria have developed mechanisms of resistance to hydrolysis by lysozyme: the production of lysozyme inhibitors. A gene product named "Ivy" for "Inhibitor of vertebrate lysozyme" has been shown to express a strong anti-lysozyme activity on *E. coli* (Monchois et al., 2001). This product could form complex with lysozyme resulting in the occlusion of the lysozyme active site by a loop protruding from the Ivy molecule (Abergel et al., 2007). Deckers et al. (2008) showed that inactivation of Ivy gene in *E. coli* render this microorganism more susceptible to the antimicrobial activity of egg white compared to the wild strain. The ivy gene was identified in the genomes of many Gram-negative bacteria and many proteins from the same family have been reported since then. Considering the widespread occurrence and effectiveness of lysozymes as antibacterial agents, it is not surprising that bacteria have in turn evolved mechanisms to resist.

In the last two decades, evidence has been accumulated for the existence of alternative mechanisms of bactericidal action of lysozyme, which are independent of its enzymatic activity.

In avian egg, the rise in the pH of the egg white begins once the egg is laid (Cf III.1). The pH increases with the time from 7.6 to 9.5 (Burley and Vadehra, 1979). Ibrahim et al. (1994) showed the absence of lytic activity of lysozyme at pH 9 (pH of the egg a few days after laying); so the lysozyme could quickly lose its lytic activity after laying. At this range of pH, lysozyme is enzymatically inactive but substantially bactericidal (Wang and Shelef, 1991). When hen eggs are stored, an irreversible dimeric form of lysozyme is generated and involved the active site. So it is still not certain that the enzymatic activity of lysozyme makes a large contribution to the defense against infections.

Another indication is that lysozyme denatured by heat or by reducing agents such as dithiothreitol (DTT), which should impair its enzymatic activity, still has bactericidal properties against some bacteria (During et al., 1999). More convincing evidence was provided by Ibrahim et al., (2001) who employed site-directed mutagenesis to inactivate the enzyme. This mutation rendered the enzyme completely catalytically inactive but did not eliminate the bactericidal properties of lysozyme against *Bacillus subtilis* and *Staphylococcus aureus*. Moreover, some have reported that peptides derived from lysozyme and devoid of the enzymatic activity can disrupt membranes (Masschalck et al., 2002). The correlation between these disturbances and cell death strongly suggests that the cytoplasmic membrane is the primary target of lysozyme. The membrane disruptive activity seems dependent on the energy status of the membrane, which is consistent with the effect observed for other cationic molecules.

Another mechanism of lysozyme antimicrobial activity was also attributed to the induction of autolysins in *Bacillus subtilis* (Ibrahim et al., 2001), a phenomenon that had been observed earlier in

oral streptococci exposed to lysozyme (Laible and Germaine, 1985). Autolysins are peptidoglycan hydrolases that bacteria use to introduce breaks in the peptidoglycan chains at the point of cell wall growth but that, under particular conditions, are able to cause bacterial autolysis.

In conclusion, it is clear that lysozyme is involved in egg white activity via several different bactericidal mechanisms. However, the relative contribution of these mechanisms is not completely elucidated. The first and the well-known mechanism is based on the enzymatic peptidoglycan hydrolysis which is characterized by spheroplast formation and possibly bacteriolysis in case of osmolarity changes. The second mechanism is non-lytic and is based on membrane disturbance. A third mechanism involves the induction of autolysins. Depending on the type of bacteria (composition and structure of the membranes and the cell wall, presence of a factor inhibiting lysozyme activity) and the environmental conditions that can affect the energisation status of the bacterial membrane or the lysozyme structure, one of these mechanisms may predominate on the others and cause cell death or act in synergy.

I.2. Ovotransferrin

Ovotransferrin is a protein chelating metal ions and belongs to the family of transferrins that have members in various animal fluids (blood transferrin, lactoferrin from milk). By creating an iron deficient environment, it has a bacteriostatic effect on bacteria. Ovotransferrin is a glycoprotein with a molecular weight of 77,700 Da containing two iron binding sites. Like all transferrins, ovotransferrin is composed of two homologous lobes. Each lobe has the ability to bind ferric iron with extremely high affinities, in the 10^{20} M^{-1} range (Abdallah and El Hage Chahine, 1999).

A large number of independent authors have found that ovotransferrin has a bacteriostatic effect on Gram-negative bacteria and that this effect is blocked if the protein is saturated with iron (Lock and Board, 1992; Garibaldi, 1970; Baron et al., 1997). The major hypothesis advanced for this activity has been that ovotransferrin produces an iron-deficient environment. Iron is essential for all forms of life. It participates in many major biological processes (Andrews et al., 2003, for a review). The iron content of egg white was generally reported to be insufficient to allow ovotransferrin saturation (Thapon and Bourgeois, 1994).

It has been repeatedly shown that the bacteriostatic action of egg albumen against Gram-negative bacteria in general is reduced by the addition of iron in amounts sufficient to saturate ovotransferrin (Lock and Board, 1992). Further studies (Garibaldi, 1970; Baron et al., 1997) showed that the major role in preventing growth of Gram-negative spoilage bacteria in egg white is acted by ovotransferrin and iron deficiency.

But many bacteria deposit intracellular reserves of iron within iron storage proteins. These iron stores can then be used to enhance growth when external iron supplies are restricted (Andrews et al., 2003). Moreover, certain bacteria can synthesise and secrete high-affinity extracellular ferric chelators called siderophores in response to iron restriction (Neilands, 1995, for a review). Siderophores have a low molecular mass (< to 10,000 Da) which solubilizes exogenous iron, making it available for transport into cells. Pathogenic bacteria are able to counter the iron restriction imposed in biological fluids through the use of siderophores that can compete with iron-binding proteins for iron.

Gram-negative bacteria take up ferri-siderophore complexes via specific outer membrane receptors in a process that is driven by the cytosolic membrane potential. Periplasmic binding proteins transport ferri-siderophores from the outer membrane receptors to cytoplasmic membrane ATP-binding cassette permease that, in turn, delivers the ferri-siderophores to the cytosol where the complexes are probably dissociated by reduction. Gram-positive bacteria use binding-protein dependent on the ATP-binding cassette. This binding protein is generally a lipoprotein linked to the external surface of the cytoplasmic membrane. However, it is possible for pathogens to acquire iron directly from iron binding proteins by using receptor-mediated transport systems specific for host iron complexes (Andrews et al., 2003, for a review).

Several lines of evidence have suggested that transferrin, lactoferrin and ovotransferrin may have a direct effect on Gram-negative bacteria in addition to those resulting from nutritional deprivation of iron.

While hydrophobic lipopolysaccharide/lipopolysaccharide or lipopolysaccharide/protein interaction contributes to maintenance of outer layer integrity, the presence of divalent cations within the membrane appears to be critical to stabilizing the strong negative charge of the lipopolysaccharide

molecules. By binding these membrane-associated cations, metal binding protein could have an effect on the membrane (Ellison et al., 1988).

Several authors (Valenti et al., 1986; Baron, 1998) suggest that ovotransferrin antimicrobial activity can result from a direct effect on the membranes: interaction of the ovotransferrin with the anionic outer membrane of Gram-negative bacteria can be hypothesized due to the remarkable cationic nature of ovotransferrin. This effect was also eliminated with ovotransferrin iron saturation.

Aguilera et al. (2003) described a molecular mechanism common to all transferrins. Transferrins are able to permeate the outer membrane of *E. coli* to access the inner membrane where they cause permeation of ions in a selective manner. The consequence is an *in vivo* dissipation of delta-phi without a significant alteration of delta-pH and a decrease in the electrochemical potential from minus 198 mV to -56 mV. As a result of the uncoupling of the respiration-dependant energy production, bacteria enter bacteriostasis.

In conclusion, there is further evidence that ovotransferrin possesses distinct mechanisms of bacteriostatic action against bacteria. The first is the well-known mechanism based on the chelation of iron that creates an iron-deprived environment for bacteria. The second is a direct interaction with the membrane resulting in the damage of the biological functions of bacterial cytoplasmic membrane. The two mechanisms are dependent on the concentration of iron in the medium: iron saturation of ovotransferrin eliminates the bacteriostatic effect.

I.3. Proteinase inhibitors

The egg white contains high amounts of active protease inhibitors, called antiproteases. Proteases are proteolytic enzymes catalysing the cleavage/degradation of peptidic chains and are involved in many biological processes. The low digestibility of raw egg white illustrates the abundance of egg white protease inhibitors, which are able to inhibit digestive enzymes. Although the reason for such a massive presence in egg white remains still unclear regarding the physiological function of the egg to support the development of an embryo, it is believed that they might be involved in the defence of the egg against bacterial contaminations. Indeed egg antiproteases could potentially inhibit proteases-secreting bacteria and/or have a direct bactericidal activity. Some bacteria are able to secrete extracellular proteases (e.g. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus*, *Porphyromonas gingivalis*...) that are considered as virulent factors due to their role in the survival, growth or invasive potency of bacteria and playing critical role in the host-pathogen interactions. The proteolytic activity of these bacterial enzymes can be counteracted by at least four of the main representative active protease inhibitors in the egg white, namely ovomucoid, ovomucoid, ovostatin and cystatin.

Ovomucoid represents the second more abundant protein of the egg white (13%) after ovalbumin (>50%). This protein (28 kDa, pI 4.1) is highly glycosylated and contains 9 disulfide bonds. The glycosylations are composed of both sialyloligosaccharides and sulphated oligosaccharides, and constitute approximately 25% of ovomucoid. This glycoprotein has a protease inhibiting activity (anti-trypsin, anti-chymotrypsin). It contains three functional homologous Kazal-like domains, each containing a reactive site. The antibacterial role of ovomucoid and its participation in the protection of egg white against bacterial contamination have not been clearly demonstrated. However some bacterial proteases like subtilisins and *Streptomyces griseus* proteases A and B are inhibited by Turkey ovomucoid (Ardelt and Laskowski, 1985).

Ovoinhibitor (1.5% of total egg white proteins) is a 48 kDa glycoprotein possessing seven kazal-like domains and 21 disulphide bonds. Each domain contains an inhibitory site. Ovoinhibitor has been shown to inhibit several serine proteases such as trypsin, chymotrypsin and elastase. Subtilisin, a serine protease produced by *Bacillus spp*, is also inhibited suggesting that ovoinhibitor has antimicrobial activity. The antimicrobial activity of yolk ovoinhibitor has been recently revealed on *Bacillus thuringiensis* (Bourin et al., 2011).

Ovostatin (or ovomacroglobulin) is a large size homotetrameric protein with four disulphide linked subunits (4 x 165 kDa) and belonging to the alpha2-macroglobulin family. This protein (0.5% of total egg white proteins) is known to bind and inhibit various proteases from different classes (serine-, cysteinyl-, metallo- and aspartyl-proteases) but with a better efficiency towards metalloproteases (collagenase, thermolysin, stromelysin). Ovostatin can be considered as an antimicrobial due its ability

of inhibiting proteases produced by several virulent bacteria inducing corneal tissue damages (*Serratia marcescens*, *Pseudomonas aeruginosa*) (Molla et al., 1987).

Cystatin is a non glycosylated protein (13 kDa) containing two disulfide bonds and representing 0.05% of total egg white proteins. This antiprotease targets most of cysteine proteases including ficin, papain, and cathepsins B, H, L. Cystatin exhibits antibacterial activity by preventing the growth of group A *streptococcus* (Bjorck 1990), *Salmonella* Typhimurium (Nakai 2000), and *Porphyromonas gingivalis* (Blankenvoorde et al., 1996). Bactericidal activity has been observed for cystatin at low dose against *Acinetobacter Iwofii*, *Escherichia coli*, *Oligella sp* and *Pseudomonas aeruginosa* (Wesierska et al., 2005).

I.4. Others

Avidin is a cationic homotetrameric glycoprotein representing 0.05% of the total protein content of egg white. This protein has a very high affinity for biotin (vitamin B8/H), and is therefore supposed to inhibit the growth of biotin-requiring micro-organisms. In addition, avidin has been found to bind to various Gram-negative and Gram-positive bacteria including *Escherichia coli*, *Klebsiella pneumonia*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermis* (Korpela et al., 1984).

Defensins are small (2-6 kDa) cysteine-rich cationic proteins involved in the host innate defense and found in many living species (vertebrates, invertebrates, plants). These molecules are characterized by conserved cysteines involved in disulfide bonds making them extremely compact and stable. Most of these molecules possess a broad spectrum activity directed against Gram-positive and Gram-negative bacteria, but also against fungi and viruses. Proteomic analysis of chicken egg white (Mann, 2007) revealed the presence of two beta-defensin related molecules: the avian beta-defensin 11 (AvBD11) and gallin. **AvBD11** is a long size beta-defensin (9,2 kDa) containing 12 cysteines involved in 6 disulfide bonds and has been shown to inhibit the growth of *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* (Hervé-Grépinet et al., 2010). **Gallin** (4,7 kDa, 3 disulfide bonds) is a member of ovodefensins, a sub-family of beta-defensins found in the egg white from different avian species (chicken, turkey, swan, duck). The recombinant form of the peptide has been shown to inhibit *Escherichia coli* (Gong et al., 2010).

OVAX (ovalbumin related protein X) is a glycosylated heparin-binding 45 kDa protein belonging to the ov-serpin family, which includes the major egg white protein ovalbumin. Serpins are serine protease inhibitors but to date, no cognate proteases have been identified for the ovalbumin and related proteins. OVAX amount in egg white is estimated to be 100-times less concentrated than ovalbumin. In spite of high sequence identity between OVAX and ovalbumin, OVAX has recently been shown to be antimicrobial for two foodborne disease pathogens, *Listeria monocytogenes* and *Salmonella* Enteritidis, whereas ovalbumin has no antibacterial activity (Réhault-Godbert et al., 2013). A patent for the use of OVAX as an anti-*Listeria* agent has been deposited (Réhault-Godbert et al., 2011). Its activity may be related to the ability of OVAX, but not ovalbumin, to bind to the anionic surfaces/molecules such as the glycosaminoglycan heparin. Indeed, heparin by blocking the heparin-binding site of OVAX is able to inhibit anti-*Salmonella* properties of OVAX, presumably by competing with bacteria at the antibacterial site of OVAX. A cluster of cationic amino-acid residues present at the surface of OVAX is supposed to interact with heparin and take part in the antibacterial mechanism (Réhault-Godbert et al., 2013).

Tenp (~49 kDa) is a protein belonging to the BPI / LBP (Bactericidal Permeability Increasing protein / Lipopolysaccharide binding protein) family. Members of this family are able to bind bacterial lipopolysaccharide, a surface component of Gram-negative bacteria, and mediate alterations of the outer membrane and damages of the inner membrane of bacteria. These events lead to the inhibition of bacterial growth (Elsbach and Weiss, 1998). By analogy with other members of this family, Tenp is supposed to be antibacterial but further investigations on this protein (purification, characterization) need to be performed to confirm Tenp predicted function.

II. Influence of environmental and physico-chemical conditions

The bacterial growth in egg white and its antimicrobial activities under the control of its proteins and molecules are influenced by different physico-chemical parameters, such as those related to environmental conditions (temperature) or the freshness of eggs (viscosity, pH). Other intrinsic factors such as CO₂ and divalent cations may also affect bacterial growth. Depending on these factors, the antimicrobial activity of egg white can be either bacteriostatic or bactericidal.

II.1. Temperature

It was clearly shown that the temperature plays a key role on the growth and survival of bacteria in the egg white. For *Salmonella* Enteritidis, the major pathogen of egg, the growth of *Salmonella* seems to be impossible at temperature below to 8°C (Ruzickova, 1994; Schoeni et al., 1995). Moreover, a bactericidal effect is observed with the disappearance of the whole population at 4°C in a few days (Chen et al. 2005; Schoeni et al. 1995; Lock and Board, 1992). Between 20°C and 30°C, a growth of one to four logarithmic units per ml, depending on temperature and incubation time is observed (Baron and Jan, 2010, for review). For temperatures above 37°C, a bactericidal effect was observed depending on temperature and incubation time (Clavijo et al. 2006; Kang et al. 2006; Alabdeh et al., 2011). In agreement with Keller et al. (1995) and Alabdeh et al. (2011) who observed poor survival of *Salmonella* Enteritidis in forming eggs or in egg white incubated at 42°C, respectively, we think that hen's body temperature (around 40°C - 42°C) enhances the antimicrobial activity of egg white proteins during the period of egg formation in the oviduct. This effect at 42°C is in connection with the egg white since in optimum medium, the temperature of 42°C is bacteriostatic but allows the survival of *Salmonella* Enteritidis.

Given the results obtained at low temperature, the storage of eggs at refrigeration temperature represents an effective way of prevent bacterial growth. Moreover, the refrigeration temperature enables to reduce the loss of the vitelline membrane integrity and the access of bacteria to egg yolk in eggshell.

II.2. Egg white pH

After laying, the loss of carbon dioxide changes the pH value from 7.6 to about 9.5 in a few days (Cf III.1.). This increase in pH value has consequences on bacteria and on egg white molecules. The maintenance of intracellular pH around its optimum value (7.6-7.8) is essential for many biological functions, particularly for bacterial enzyme activities and status of the membranes. The alkaline pH mainly affects the respiratory status of the cells that are trying to preserve the proton motive power and their electrochemical potential. To do this, bacteria accelerate the importation of protons and block their export. They also suppress the energy cost systems such as those associated with flagellar biosynthesis and chemotaxis (Maurer et al., 2005). Baron (1998) has indeed shown that *Salmonella* Enteritidis lacks flagella at alkaline pH, which may limit its access to nutrients.

Concerning the effect of alkaline pH on egg white proteins, it has been suggested that alkaline pH may promote the antimicrobial activity of ovotransferrin by interfering with bacterial iron metabolism, preventing bacteria from obtaining sufficient iron for growth in the egg white, an environment already deficient in iron (Tranter and Board, 1984). A higher activity of ovotransferrin was recorded at alkaline pH than at acidic pH, due to an acceleration of iron release under acidic conditions and a slowdown under alkaline conditions (Halbrooks et al. 2005; Okamoto et al., 2004). Recently, Levashov et al. (2010) also showed that the lysozyme activity was increased under alkaline pH. However, according to others (Ibrahim et al., 2001), the lysozyme peptidoglycan lytic activity is inhibited by alkaline pH without change in its other bactericidal activities. Otherwise, the activity of some antimicrobial peptides could be raised at alkaline pH (Abriouel et al., 1998).

The role of alkaline pH on the antimicrobial activity of egg white has been highlighted by several studies (Kang et al., 2006; Messens et al., 2004; Tranter and Board 1984). These studies show that at pH greater than 8.8, the egg white shows bacteriostatic or bactericidal effect in contrast to pH 7.5 or 8 that allow bacterial slight growth or bacteriostic activity.

II.3. Egg white viscosity

The viscosity of the bacterial environment is a physical parameter affecting the motility of flagellated bacteria (Schneider and Doetsch, 1974). The egg white is a viscous fluid composed of three distinct layers surrounding the egg yolk: the outer thin egg white, the thick egg white and the inner thin egg white, respectively from the exterior to the interior of the egg. The viscosity of egg white probably makes difficult the motility of bacteria and the access to nutrients required for bacterial growth. Due to its high viscosity, the thick egg white may be considered as a physical barrier, like the egg shell and membranes. The decrease in the egg white viscosity observed during the egg storage may help bacteria to spread in the egg. Some authors have shown that the growth of *Salmonella* is favoured at 20°C for eggs stored beyond 3 weeks (Dubocage et al., 2001; Messens et al., 2004) probably due to the decrease in viscosity that would favour either the passage of nutrients from yellow to white or migration of bacteria to the yellow (Humphrey and Whitehead, 1993; Chen et al., 2005).

The egg white viscosity is attributed to the presence of ovomucin and more particularly to the complex it forms with lysozyme. The viscosity depends on several factors: the laying hen line, the fraction of egg white involved (thin versus thick egg white), time and storage conditions. Thus, the viscosity of egg white decreases during storage of eggs (Cf III.1.), the white gradually liquefies and loses its gel structure. This is typically explained by the dissociation of the complex ovomucin-lysozyme due to the increase in pH. This leads to the weakening of the electrostatic bonds of the complex. Although the egg white thinning probably favours bacterial spreading in the egg, this effect may be counterbalanced at least in part by the loss of flagella of bacteria due to alkaline pH.

Ovomucin is a sulphated, high molecular weight, highly glycosylated protein belonging to the family of mucins, a group of proteins found in the numerous secretions of epithelial tissues and involved in the gel-like properties of secretions in which they are present. The egg white is a biological fluid secreted by the tubular gland and epithelial cells of the magnum to protect the egg yolk, and numerous studies have suggested that ovomucin has an important role in the gel-like structure of the egg white, although its function in the avian egg and during the embryo development remains still unclear. Its interaction with lysozyme at neutral pH is thought to alter antibacterial properties of lysozyme. Mucins have been shown to inhibit the muramidase activity of lysozyme (Park et al., 2006) and alter antibacterial activities of numerous antibacterial proteins/peptides. So it is likely that ovomucin may have similar effects on egg white antimicrobial molecules, and that the dissociation of lysozyme from ovomucin at alkaline pH observed during the egg white thinning could release additional antibacterial activities. Thick and thin egg whites which are characterized by different ovomucin composition and/or properties, may also display distinct behaviours in terms of antibacterial activities. For instance, it has been shown that the growth of *Salmonella* Typhi is favoured in the thick egg white compared to the thin egg white (Yadav and Vadehra, 1977). This may be due to the different structure of ovomucin between these two fractions since the addition of thick egg white ovomucin to the thin egg white has been shown to interfere with the antibacterial activity of thin egg white (Yadav and Vadehra, 1977).

II.4. Other parameters

The egg forms in the oviduct in a carbon dioxide (CO₂)-rich environment. As a consequence, the egg white from freshly laid eggs contains dissolved CO₂ which progressively escapes through the eggshell to the external milieu during egg storage (Cf III.1.). Carbon dioxide is able to inhibit the bacterial growth of aerobic microorganisms by affecting the lag phase and the growth rate. This effect has been observed for several spoilage bacteria (Devlieghere and Debevere, 2000; Gill and Tan, 1980). A higher effect of CO₂ is generally observed for Gram-negative bacteria by comparison to Gram-positive bacteria which are mostly facultative or strict anaerobes. Strict anaerobes seem not to be affected by CO₂. Carbon dioxide was found to inhibit the growth of *Enterobacteriaceae*, including *Salmonella* (Phillips, 1996). However, a recent study suggests that *Salmonella* Enteritidis, when inoculated in egg white, may resist to its inhibiting effect (Gurtler and Conner, 2009). Interestingly, besides its direct effect on bacteria, CO₂ can also potentially interact with protein activities, as demonstrated for lysozyme whose lytic activity is modulated by CO₂ (Banerjee et al., 2011).

Ionic composition of the egg white is also of particular interest. The absence of free iron (due to large amounts of ovotransferrin) makes the egg white quite inhospitable to support the growth of iron-

requiring bacteria (Cf I.2.). Egg white also contains divalent cations such as magnesium and calcium with potential effect on the efficacy of egg antibacterial components. Divalent cations (even at subphysiologic levels) have been shown to affect the activity of mammalian cationic antimicrobial peptides, as previously demonstrated for LL-37 (Deslouches et al., 2005). They are able to bridge lipopolysaccharides on bacterial surfaces, suggesting that they may serve as competitive inhibitors to cationic antimicrobial molecules. Interestingly, Mg^{2+} and Ca^{2+} were found to inhibit the potent bactericidal activity of a heat-denatured form of egg white lysozyme (without muramidase activity) against *Staphylococcus aureus* and *Escherichia coli* K12 (Ibrahim et al., 1997). The effect of divalent cations on native egg white antibacterial proteins remains to be further investigated.

III. Potential applications regarding egg storage conditions

Shell eggs are generally consumed within days or weeks after laying. During this period, eggs are stored at various temperatures depending on the place of storage (laying farm, conditioning centre, food store, consumer's home...) and might be subjected to bacterial contaminations. In the conditioning centre, the storage temperature is ideally comprised between 5°C and 20°C (Buffet, 2010). At consumer's home, eggs are usually either refrigerated or stored at room temperature if not immediately consumed. The storage of eggs between laying and consumption induces internal modifications, which are strongly influenced by temperature and duration of storage. Physicochemical modifications and antibacterial properties of the egg white during egg storage will be successively described in this part of the review. Innovative methods for egg storage illustrated by the use of carbon dioxide will be also discussed in relation to the quality and safety of shell eggs.

III.1. Physicochemical modifications of egg white during egg storage

During egg storage, albumen undergoes important modifications, the most dramatic change being the thinning of the thick white. These modifications are due to gas exchanges between the interior of the egg and the external milieu through the eggshell, and exchanges between the egg white and the egg yolk through the vitelline membrane (Thapon and Bourgeois, 1994, for review). Time and temperature are two factors having crucial roles on the evolution of egg white during storage.

Water and carbon dioxide are two egg white components leaking out of the egg through eggshell during egg storage. The water loss depends primarily on the integrity of cuticle. Due to the progressive degradation of the cuticle after the egg is laid, the water loss increases over time during egg storage and can be dramatically enhanced when eggs are washed. Water elimination through eggshell is affected by several parameters including storage temperature, relative humidity, storage time, eggshell surface and eggshell porosity. At oviposition, albumen contains dissolved carbon dioxide (4-5 mg/egg) and combined carbon dioxide present in bicarbonate (about 100 mg). During egg storage, carbon dioxide extricates and leaks through eggshell pores to the external milieu. The rate of CO_2 leakage through the eggshell is function of storage temperature. The loss of carbon dioxide observed during egg storage has two remarkable consequences on the albumen properties: pH increase and egg white thinning, both being dependent on storage temperature. The egg white pH value varies from 7.4 at laying to 9.5 within a few days post-laying. The pH increase can be inhibited or limited by preventing CO_2 leakage out of the egg. The obstruction of pores (by using substances like oil, wax, silicates...) in freshly laid eggs and the incubation of eggs in a CO_2 -enriched atmosphere (Cf III.3.) are methods allowing to restrain pH increase in egg white. In this case, egg white pH relies on CO_2 content. Egg white thinning in stored eggs is characterized by a decrease in the viscosity of thick egg white. This phenomenon is accelerated by increasing storage temperature. Several hypotheses have been proposed to explain the molecular mechanism of egg white thinning. Here are some of them:

- Dissociation of lysozyme-ovomucin complexes resulting from electrostatic interactions at alkaline pH (Cotteril and Winter, 1955).
- Dissociation of ovomucin components, in particular a solubilization of the β component, rich in glucids, which transfers to the thin albumen (Hayakawa et al., 1983). The fact that a part of divalent cations (Ca^{2+} and Mg^{2+}) transfer from the albumen to the yolk during storage accelerates this phenomenon.

- Partial degradation of O-glycosidic bonds following the solubilization of β -ovomucin and release of carbohydrates (hexoses, hexosamine, sialic acid) by β -elimination at alkaline pH (Kato et al., 1979).

Due to the difference of osmotic pressure from both sides of the vitelline membrane and to the lower hydration of yolk proteins, a transfer of water and minerals from the albumen to the yolk can be observed during egg storage. It is accompanied by sodium fluxes while potassium is less affected. This phenomenon affects primarily divalent cations such as calcium and magnesium. Their contents decrease rapidly in albumen and increase in the yolk. This loss in divalent cations is also supposed to contribute to the egg white thinning.

III.2. Modulation of egg white antibacterial properties during egg storage

Bacterial penetration and growth in egg white depends firstly on the egg integrity but also on the conditions of egg storage (time and temperature) (Baron and Jan, 2011, for review). The growth of *Salmonella* is favoured at 20°C for eggs not older than one week, as compared to eggs stored at this temperature for 2-3 weeks (Dubocage et al., 2001). Beyond three weeks, the growth may be stimulated. These favourable conditions for *Salmonella* multiplication are likely to be related to the alteration of the vitelline membrane and to the decrease in egg viscosity, both being due at least in part to the dissociation of the ovomucin-lysozyme complex at alkaline pH. Both factors favour the entrance of nutrients from egg yolk to egg white and the migration of *Salmonella* into the yolk (Humphrey and Whitehead, 1993; Chen et al., 2005).

Besides analyses of *Salmonella* growth in stored shell eggs, the evolution of the egg white antibacterial potential during egg storage has recently been studied using egg white samples isolated from shell eggs stored for different time at variable temperature (Réhault-Godbert et al 2010). In this study, the antibacterial potential of each egg white sample has been assessed by measuring *in vitro* the growth of *Salmonella* Enteritidis at 30°C for 3 days. Results showed that egg white antibacterial activity is significantly increased within few days following oviposition. The activation of these anti-salmonellic defenses is strongly influenced by storage time but also by temperature. Indeed, anti-microbial properties in the egg whites sampled from eggs stored at 37°C is progressively increased between 0 and 5 days following oviposition; it is then progressively altered after two weeks of storage. This alteration might be partially explained by the degradation of ovotransferrin which is the major anti-*Salmonella* agent in egg white. When the egg is stored at 20°C, the activation of egg white anti-microbial properties is more progressive being maximal after ten days but then stabilizes up to one month after oviposition. These observations suggest that egg white thinning and/or increase in pH in egg white may be involved in the activation of anti-salmonellic properties of albumen during storage, at least during the first week of storage. Considering the dual effect of high temperature during egg storage, favouring bacterial growth and the putative antibacterial activity of egg white, further experiments need to be conducted on shell eggs internally inoculated with *Salmonella* Enteritidis to find the conditions minimizing *Salmonella* growth during storage, one possibility being to introduce a short period of higher temperature to stimulate the antibacterial activity of egg white.

In conclusion, these observations altogether suggest that some conditions of egg storage can reinforce the antibacterial potential of the egg white, at least against *Salmonella*. This activation has been observed for *Salmonella* Enteritidis but it is not known if it occurs for other bacterial species. The underlying molecular mechanisms remain unclear, although egg white thinning and/or pH increase are likely to be involved. These observations underline the interest to applying temperature sequences. For instance, applying a moderate to high temperature to shell eggs for a short period (activation of anti-Salmonellic defences) and then cool down eggs to keep both freshness and hygienic quality of egg white (Cf part II.1). A pulse of high temperature can be performed by pasteurization. This method is used in United States to decontaminate shell eggs. However, the effect of such a treatment on the antibacterial activity of egg white and proteins remains to be investigated. Warm/hot temperatures applied to shell eggs can weaken the vitelline membrane, particularly in case of prolonged treatment. This degradation, also observed during storage at room temperature, may facilitate the passage of *Salmonella* from the egg white to the yolk. Refrigeration of shell eggs could stop or limit the heat-induced degradation of the vitelline membrane. In practice, it is noteworthy that freshly laid eggs nowadays are not refrigerated but stored under cool/mild temperature conditions within days before

reaching the consumer's home. The refrigeration of shell eggs immediately after laying should be strongly encouraged because of its beneficial effect on the vitelline membrane integrity.

III.3. Use of CO₂ during of egg storage

One way to control egg white properties during storage is the use of modified atmospheric packaging (MAP). Modified atmospheres are used to extend the shelf life of foods by inhibiting chemical, enzymatic and microbial spoilage. The gases used in most MAP applications include CO₂, O₂, N₂ or different combinations of these gases mainly used to preserve fruits and meat during storage. Physico-chemical and microbiological properties of eggs packed in air, 100 % O₂, and 100 % CO₂ and stored up to 45 days at 4, 20 and 36 °C were evaluated during the RESCAPE European program (2006-2009). The weight loss of eggs classically observed during egg storage was significantly decreased for all packed eggs. In normal storage conditions, the pH of albumen progressively increases up to 9.5 and the Haugh units decrease in relation to the egg white thinning, but the use of Air and O₂ MAP stabilizes egg white pH and slows down the decrease in Haugh units when eggs are stored over a period of 28 days at 25°C (Rocculi et al., 2009). A more spectacular effect is observed when using CO₂ MAP which improves the physical-chemical egg white quality (decreased pH, stabilized Haugh units) (Rocculi et al., 2009). MAP technology was also tested on the survival of experimentally inoculated pathogen bacteria (*Salmonella* Enteritidis) during the storage at various temperatures (Pasquali, Manfreda, Sirri, personal communication). Compared to 100% O₂ or Air MAP, no negative effect was observed for 100% CO₂ MAP regarding the number of *Salmonella* Enteritidis in egg white when combined with chilled conditions over a period of 45 days. A deleterious effect was however detected at higher temperature (20°C) since a higher load of *S. Enteritidis* was detected in the egg white issued from 100% CO₂ eggs, by comparison to eggs packed with 100% O₂ and air.

The rapid cooling of shell eggs using cryogenic CO₂ has also been used to improve the quality and safety of eggs. This method is patented since 2000 (Curtis et al., 2000). Microbial counts were carried out in inoculated eggs submitted to cryogenic CO₂ cooling or traditional air cooling. Reduced *Salmonella* number is detected in the egg white of cryogenically CO₂ cooled eggs, by comparison to traditional air cooling. Interestingly, eggs rapidly cooled by CO₂ have not only a better hygienic quality but also a longer shelf life given its effect on Haugh units and pH.

Conclusions

In conclusion, the egg is a complex antimicrobial biological fluid. The most well-known antimicrobial proteins of egg white are lysozyme, ovotransferrin and protease inhibitors but several minor antimicrobial proteins have recently been characterized. It is likely that egg white contains still unknown proteins or peptides involved in the bactericidal or bacteriostatic activities. The coexistence of a multitude of antibacterial compounds in the egg white suggests that there may be interactions between these molecules or synergistic effects. In addition to the presence of these molecules, physicochemical/environmental conditions have also been demonstrated as playing an important role in the bacterial growth and in the egg white antimicrobial activity. Extrinsic (temperature) and intrinsic (alkaline pH, viscosity, CO₂, ionic composition...) factors can affect either bacteria or egg white molecules, leading to enhanced or decreased capacity of bacteria to grow and survive in egg white. Even if it is difficult to know the part play by each factor and their interaction, it seems that egg storage conditions through pH and temperature strongly influence the potential of egg white antimicrobial activity. Egg white undoubtedly has a variety of mechanisms allowing for the control of bacterial growth, depending on the type of bacteria and the environmental conditions. One of these mechanisms may predominate for a particular bacterium. In this regards, it clearly appears that the iron deficiency due to ovotransferrin plays a major role in the egg white activity against *Salmonella* Enteritidis. Other bacteria appear insensitive to this activity. For example, *Listeria* is mainly inhibited in the egg white by lysozyme together with alkaline pH. The egg white has a highly selective effect on microorganisms: the high incidence of *Salmonella* Enteritidis in the egg content could be explained by the fact that this bacterium has advantages for survival in egg white or for the colonization of the hen reproductive tract. At last, it is noteworthy that internal egg antibacterial activities are not inert but

evolve over time according to extrinsic environment factors (temperature, atmosphere). Although it adds further complexity to the current knowledge, the modulation of antibacterial properties during egg storage is a very interesting mechanism with a promising potential to optimize hygienic quality of shell eggs. Understanding the mechanisms of egg white antibacterial defences with an emphasis on *Salmonella* Enteritidis is a prerequisite to develop innovative and efficient systems for safe eggs preservation.

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