RESCAPE

Reducing Egg Susceptibility to Contaminations in Avian Production in Europe

Project number: 036018

Specific Targeted Research project
Sixth Framework Programme
Thematic Priority 5
Food quality and safety

Final Publishable Activity Report

Period covered: From October 1st, 2006 to September 30, 2009

Date of preparation: October 2009

Revision: 1

Start date of the project: October 1st, 2006 **Duration:** 36 months

Project coordinator name: Yves Nys

Organisation: Institut National de la Recherche Agronomique (INRA)

Project execution

Summary description of project objectives

By 2012, Council Directive 1999/74/EC defining minimum standards for the welfare of laying hens will abolish conventional cage systems in favour of enriched cages or floor systems in order to improve the welfare of hens. However, the scientific AHAW (Animal Health and Animal Welfare) panel (EFSA) in agreement with BIOHAZ (organisations in charge of Biological Hazards) and CONTAM (contaminants) panels concluded that keeping hens on the floor or outside might present an increased risk of contamination due to a greater exposure to infectious agents or parasites.

The overall objective of RESCAPE is to reduce the risk factors from eggs in alternative systems of production by providing means to reduce egg contamination.

This is achieved in particular by participating in the collection of new data on the bacteriological contamination of eggs in various types of production (enriched cages and floor systems) in France in collaboration with a complementary project active in this area: Safehouse.

The second objective of the project is to propose methods of reinforcing the antimicrobial defence of eggs; improving non invasive measurement of egg quality; reducing veterinary treatments by introducing vaccination against red mite and developing new techniques for egg decontamination to reduce food safety risks generated by these systems while maintaining a high quality product. Our strategy attempts to reach perfection of production and egg sorting and decontamination system in the short term as well as longer-term genetic improvement of the antimicrobial capacity of eggs.

This will provide information to adapt, if needed Directive 1999/74/EC before the 2012 deadline. It helps producers adapt their production system without loss in profitability and satisfy European consumer demand for safer food produced in respect to welfare standards. This should help to avoid the migration of egg production from Europe towards production sites over which the EU has no control and, therefore, contribute to maintaining over one hundred thousand jobs involved in the production and selling of eggs in EU-27 and retain the current traceability of products in Europe by limiting the importation of eggs that do not have to comply with the stringent EU-27 production standards.

Contractors involved

Institut National de la Recherche Agronomique (France)
French Agency for Food Sanitary Safety (France)
Institute for Agricultural and Fisheries Research (Belgium)
GLON/ Sanders- Cybelia(France)
INRA Transfert (France)
Catholic University of Leuven (Belgium)
Microwave Energy Application Company (Belgium)
Roslin Institute (United Kingdom)
University of Bologna (Italy)
University of Glasgow (United Kingdom)
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Overview of general project objectives and current relation to the state-of-the-art

The highly competitive market of egg production with its low profit margins has resulted in the development of intensive caged hen egg production in Europe which has been considered as unacceptable to some consumers in terms of animal welfare. Consequently, Directive 1999/74/EC banned the conventional cage system and imposed its replacement by non-cage or enriched cage systems. The conventional cage system is currently predominant in Europe and has benefited from optimisation over three decades, avoiding major hygiene problem and resulting in lower costs relative to alternative system, especially compared to the outdoor system.
The report of SANCO (SPC 2003258) underlined the move in the Member States to more welfare friendly systems and the large variation in use between European countries (20 to 98% of cage production) due to market forces and differences in legislative constraints. EFSA at the demand of the EU commission expressed an opinion on welfare for hens and safety of eggs in the various production systems. Keeping hens on floor system and outdoors is considered to increase the risk of exposure to infectious agents and to favour the prevalence of downgraded eggs due to the higher level of bacterial load in the environment of hens and on eggs. Floor systems also favour presence of parasites in hens and hence increase the application of veterinary treatment, the residues of which can be found in eggs. However, there is limited information on the level of biological and chemical contamination related to the system of production. A large survey looked at two serotypes of Salmonella prevalence in numerous hen flocks of the 25 Member States and underlined the high variability between countries about the prevalence of Salmonella contamination (0 until 80 % of positive flocks depending on the countries, mainly carried out on the dominant cage system). Its reflected different environment predominantly in conventional cage production systems and various hen management practise. Legislative constrains and national control

programmes on Salmonella including slaughter of breeder hens salmonella positive flocks are likely to have largely influenced salmonella prevalence.
Recent development and commercial experience of aviaries and barn systems (mainly in Scandinavian countries and The Netherlands), free range (France, UK) or enriched cages (Sweden) led to improvements in the design of systems and management of hens and have partly solved some of the problems. Treatments of eggs such as egg washing, currently banned in Europe with exception of a few countries may help but its use remains controversial and limited in Europe.
In conclusion, there is still a lack of quantitative information over many European countries about the real influence of the system of hen housing that will be implemented in 2012 on the risk of egg contamination. Even where promising improvements have been obtained in alternative or enriched caged system, technical progress is still required to insure high quality and safe eggs are produced from these innovative egg production systems to minimise the risk of egg-borne food diseases.

RESCAPE is collaborating with SAFEHOUSE in the provision of some new quantitative experimental data on contamination to establish the risk factors in alternative systems. Microbiological analysis (Salmonella and total aerobic mesophilic flora) is carried out in various types of egg production systems quite specific to France and therefore contribute to identify hen housing and management factors which directly influence the bacteriological quality of eggs.
In addition, vaccines are very powerful tools which help to avoid the use of veterinary drugs in animals. In this project we are assessing how red mite antigens can contribute to the final development of a vaccine against this very common infestation. The success of such research is of tremendous importance for producers because at present there is no efficient treatment against acari. Indeed, large doses of acaricide are no longer allowed because of the new MRL-procedures in the EU.
Furthermore, the multidisciplinary approach adopted in this project includes several novel features in its proposal to reduce the risk of contamination from eggs. These include:
1. Using genetics and genomics to identify egg genes coding for proteins with antibacterial properties and search for markers of these genes in a commercial pedigree population to assess the relevance of their use in selection.
2. Using innovative technology that integrates new knowledge on non invasive techniques (EggDefence project) to measure egg quality in egg sorting plants
3. The development of alternative technologies to egg washing for reducing egg contamination: (1) microwave, hot air pasteurisation, and gas plasma sterilization (2) modified atmosphere packing for preservation of egg quality, (3) evaluate chemical decontamination by coating eggs with chitosan.
In conclusion, the development of these innovative technologies should contribute to optimise conditions of egg management in the short term throughout the food chain and will ensure high quality and safe eggs for consumers from the various systems of production. In parallel, research on genetics will provide major information that will be useful in the long term through hen selection.

Summary for the entire period of the work performed and main achievements

Introduction

During the whole three year period, RESCAPE has completed a majority of the initial milestones to reach its multidisciplinary objectives aimed at reducing the risk from eggs which are unfit for human consumption by developing complementary solutions at the level of the hen (breeding), farm (design and management) and egg marketing (egg decontamination and sorting).The main objectives of the project were to propose means to reduce some of the potential risk of contamination induced by new egg production systems. This has been achieved by:

- assessing egg contamination in various laying hen housing systems and to identify factors at risk (WP1)
- developing a vaccine to reduce the poultry mite survival (WP1)
- Identifying then testing a large number of gene candidates to select hens on the antimicrobial capacities of eggs using Marker Assisted Selection (WP2 and 3)
- Improving non-invasive technology to sort and identify eggs at risk (WP4)
- Explore the feasibility of using novel egg decontamination treatments (WP5)

In addition, RESCAPE (WP6) has continued to inform all stakeholders (consumers, scientists, legislators, poultry professionals) of the progress of the RESCAPE project.

Egg contamination and system of production in commercial flocks

The purpose of this approach was to assess the impact of various hen housing systems currently available in France on the egg-shell contamination and on hygiene of breeding equipment and its aptitude for decontamination. Results of studies under experimental conditions suggest a higher contamination of the eggs laid in alternative systems and in furnished cages than in conventional cages (Protais, et al., 2003a; de Reu, et al., 2005; Mallet, et al., 2006). Our objective was to assess eggshell contamination in commercial production from various laying hen housing systems in France and to identify the factors in laying hen rearing management and egg handling practices influencing this contamination. The systems studied were those currently available in France and included conventional cages, furnished cages complying with the requirements of Directive 1999/74/EC and three alternative systems : on-floor in a barn without an open-air range, free range and organic.

Twenty-eight flocks housed in cages and 30 flocks kept in on-floor systems were studied from September to October 2007 in Brittany. Each farm was visited once for collection of data on design equipment, laying hen management and egg handling practices. Sixty sorted eggs (elimination of dirty and macro-cracked eggs) were sampled to obtain counts of the total mesophilic flora (Protais, 2003b).

The mean bacterial count on eggshells tended to be higher in on-floor systems (4.82 +/- 0.51 log CFU/eggshell) than in cage systems (4.57 +/- 0.58 log CFU/eggshell, P=0.09). In the three aviaries, the mean eggshell contamination was equal to 5.52 +/- 0.57 $_{IC95\%}$ [4.11-6.92] log CFU/eggshell. The difference in contamination between eggs from on-floor flocks and eggs from cage flocks was actually significant when comparing on-floor systems to conventional cage system (P=0.03) but was no more significant when comparing on-floor systems to furnished cage system (P= 0.33). Within each type of housing system, there was no difference in eggshell contamination between free-range and organic flocks (P=0.49)

whereas in cage systems, the contamination was higher in furnished cages than in conventional cages (+ 0.6 log CFU; P=0.02). The higher contamination in furnished cages than in conventional cages may be linked to a problem of nest acceptance, especially if the eggs are laid outside the nest in a dirty part of the cage. The eggshell contamination significantly increased with the age of the hens (P<0.001), with the air dust concentration in the living area of hens (P<0.01), when the eggs were manually packed rather than automatically (P=0.04) and when eggs were packed in plastic cells in contrast with in carton cells (P=0.05). Plastic egg flats could be re-used by the farmers whereas recycled pulp trays were destroyed at the grading center after use. Inadequate disinfection of the plastic flats might explain the higher bacterial load on eggs packed in this type of packaging.

It could be concluded from the larger sample of flocks controlled that the shell contamination of eggs produced in commercial conditions, and before transport to the grading center, would not exceed 5 log CFU/eggshell, except in furnished cages and in aviaries. The present study confirms in production conditions, the effect of the housing system on eggshell contamination, previously described in experimental assays. The higher bacterial load on eggs produced in alternative systems and in furnished cages than in conventional cages might be associated with a higher aerial dust concentration but the difference in contamination remained less than 1 log CFU/eggshell. The high concentrations of dust in the ambient air of the henhouses studied also raised concern for the respiratory health of stockmen. Special attention should be paid to the hygiene and cleanliness of egg packing materials especially when these are re-used.

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Vaccine improvement for reducing treatment on hens and residues in eggs

Modern poultry production uses fully integrated techniques that allow for production of a large number of eggs in a limited amount of space and over a relatively short period of time (Axtell and Arends, 1990). Current production systems include intensive cage and extensive floor-based systems, such as barn and free-range. All of these systems are controlled to a degree, with the former being subject to severe environmental restrictions and the latter being more representative of the birds' natural surroundings. As a result, the ecology of poultry parasites is tied to the synthetic environment in which they and the birds exist and changes in this environment which negatively effect parasites are likely to be detrimental to the birds themselves (Axtell and Arends, 1990). Since the facilities and techniques for modern poultry production are fundamentally the same throughout the world, excluding climatic and geographical variability, conditions for parasites are ideal, worldwide (Axtell and Arends, 1990).

It has been suggested that the poultry red mite is currently the most economically deleterious ectoparasite of laying hens in several countries (Chauve, 1998) and has been identified globally (Levot, 1991; Axtell, 1999). The poultry red mite is an obligatory haematophagous (blood-sucking) ectoparasite of both domestic and wild birds, although it has been known to engorge on a range of other species, including man (Bruneau *et al.,* 2001). The poultry red mite is referred to as a temporary parasite, since it is only found on the host when obtaining a blood-meal, with the majority of its lifecycle spent concealed in cracks and crevices of the house substructure. Subsequently, domestic poultry systems provide the poultry red mite with a wealth of potential hiding places, particularly in barn and free-range systems (Kilpinen, 2001). Therefore, the ban on cage systems within the EU proposed for 2012 is likely to indirectly reduce the welfare of hens due to the higher prevalence of the poultry red mite in extensive systems (Höglund *et al.,* 1995; Guy and Edwards, 2006).

The poultry red mite is largely seen as a problem affecting laying hens and to a lesser degree, broilers due to rapid turnover of meat birds (Kirkwood, 1967). The poultry red mite also exists as a threat in the spread of disease, since it can act as a vector for a number of pathogenic poultry infections such as *Salmonella* spp., spirochaetosis, chicken pox, Newcastle disease, fowl typhoid, fowl cholera, amongst others (Chirico *et al.,* 2003; Moro *et al.,* 2005). However, the most profound effect of the poultry red mite is as an obligatory blood sucking parasite (Chauve, 1998). The feeding mite can cause irritation, restlessness and mild or severe anaemia, occasionally resulting in death. Subsequently this can reduce both egg production and quality, as well as compromising welfare (Urquhart *et al.,* 1996).

In Rescape, The main objective was to substitute veterinary treatments currently used against the poultry red mite *Dermanyssus gallinae* by developing a vaccine against this pest. The substitution of chemical treatment by a vaccine should reduce veterinary drug residues, mainly in the form of acaricides, in the eggs destined for human consumption. The work was carried out by the University of Newcastle (UNEW).

The approach was to use different potential vaccine antigens and seek to increase antibody and cellular responses from the poultry birds to reduce mite survival after feeding on them and prevent mite proliferation in poultry infrastructures. Crude mite antigens were initially used and showed some immune stimulation but were not very effective as the first adjuvant used was also responsible for some immune interference: Most birds showed a consistent increase in IgY level after the three successive immunisations, but without effect on mite survival. A second adjuvant gave better results on IgY production as shown in the figure below. The IgY serum response in immunized birds showed an initial peak in IgY titres on day 19 followed by a much larger peak in IgY titres on Day 43, after the second immunization.

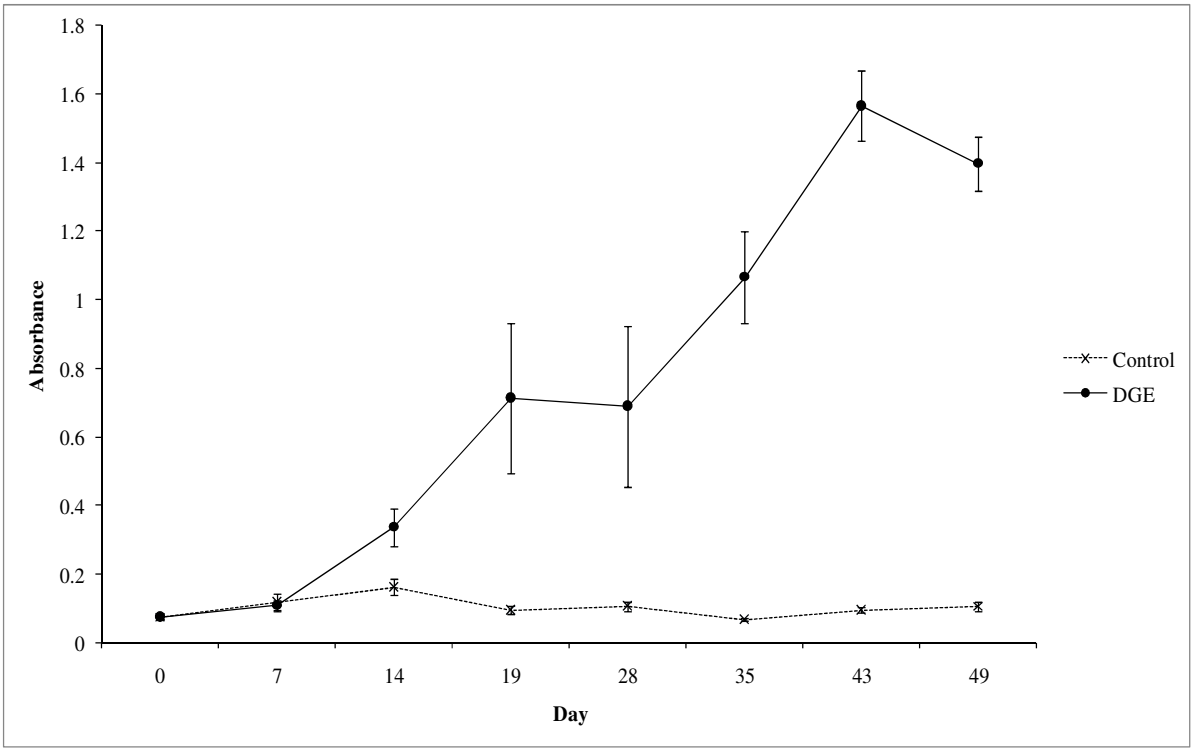

Figure 1: Serum IgY response after immunization against whole mite antigens

However the death rate in mites was still too low at below 80% and other means of vaccination were needed such as using recombinant antigens in collaboration with colleagues from the USA and Spain. Two recombinant recombinant tick proteins antigens were used and showed for one a better efficacy against the poultry red mite Dermanyssus gallinae.

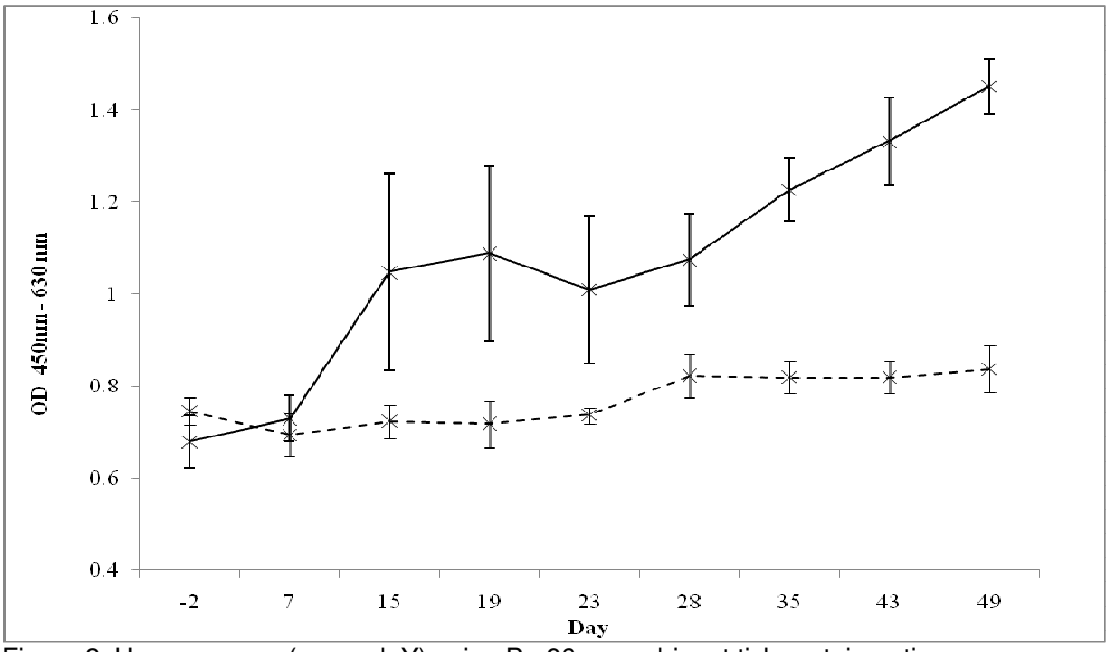

Figure 2: Hen response (serum IgY) using Bm86 recombinant tick protein antigen

IgY response to Bm86 immunization was indeed markedly different to that seen with subolesin (figure 2). IgY levels were only significantly higher in the Bm86 compared to the control treatment on Days 35, 43 and 49 ($P < 0.001$; second vaccination was carried out on Day 21). Yolk IgY levels were significantly higher in the Bm86 compared to the Control treatment on Days 35 and 43 ($P < 0.001$).

Immunization of birds with subolesin resulted in significantly higher IgY levels compared to Controls from Day 7 onwards (*P < 0.001).* IgY levels in subolesin-immunized birds peaked on Day 15 and, after a second immunization on Day 21, levels peaked on Day 28 after which they declined until Day 49 (Figure 3). No IgY response to subolesin was detected in sera from Control birds at any time point. Yolk IgY levels were significantly (*P < 0.001*) higher in the Subolesin compared to Control treatment at the three time points measured, Days 28, 35 and 43.

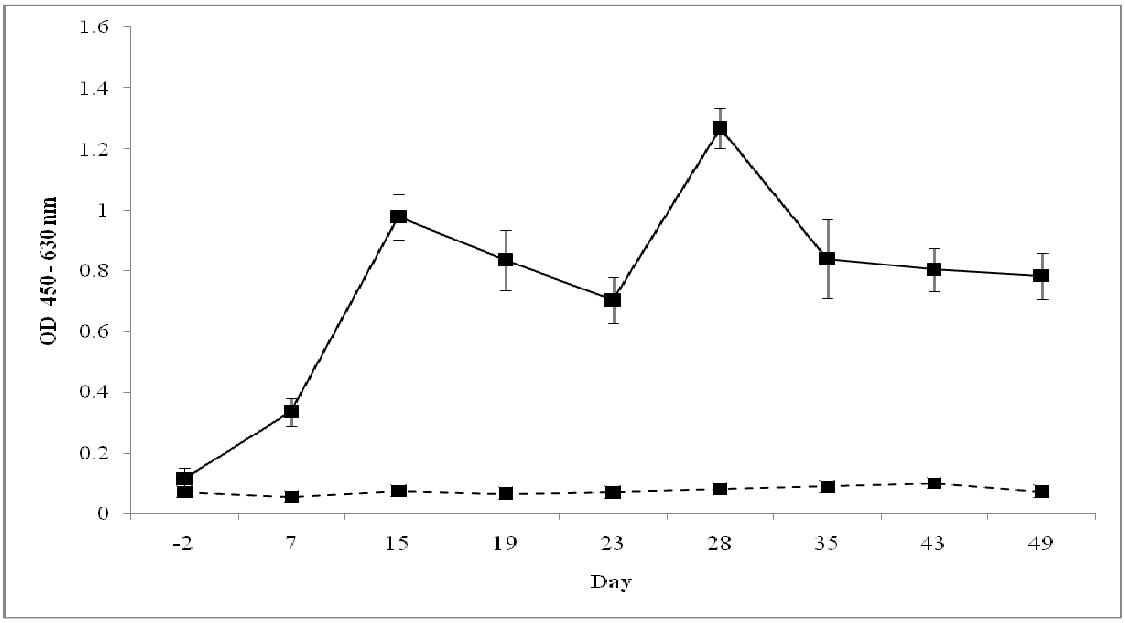

Figure 3: hen response when using the subolesin recombinant antigen

Recombinant antigens such as subolesin, compared to traditional antigens extracted from red mite populations, gave a higher mortality rate above 85%"

We also improved blood feeding techniques to obtain a higher percentage of mites feeding on the birds by using temperature cycles. We also reduced the use of animals in our experimentations by replacing animal skins with synthetic membranes during in vitro feeding trials and showing similar feeding rates.

In Conclusion, the use of recombinant antigen such as Subolesin gave the best results during the study period showing a good antibody and cellular response and the higher possible death rates for the poultry mites. Other antigens are currently used by other colleagues and could also generate good or even better results

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Introduction

The chicken egg is a natural container, which contains all the components essential for embryonic development. To ensure this function, the egg contains well-balanced nutritious elements (Nys 2001), and possesses many molecules with a broad range of activities including antibacterial, antiviral, antioxidant, cryoprotective, immunomodulating and antiadhesive activities (Mine and Kovacs-Nolan, 2006; Rehault et al., 2007). Besides their importance for embryonic development, these molecules are of major interest for pharmaceutical, cosmetic and food industries.

By 2012, the EU directive 1999/74 defining minimum standards for the protection of laying hens plans to abolish the conventional cage system in favour of furnished cages, aviaries or other floor systems, in order to improve the welfare of the hens. These modifications of the housing systems might increase the microbial charges on eggs produced for human consumption and thus risks of toxi-infection. In this context, it is important to find solutions that could prevent this issue. In this project we proposed to study in details egg's natural defences against bacteria and explore how they could be improved : (1) the eggshell that acts as a physical barrier against bacterial penetration, (2) the chemical protection composed of proteins mainly identified in the egg white but also in the yolk and the shell. Both systems are the result of endogenous egg proteins.

The objectives of the second workpackage of Rescape were therefore to identify candidate proteins with antimicrobial properties The work was carried out by INRA (Nouzilly and Rennes, France), Roslin Institute, the University of Glasgow and the University of Ottawa (Canada).

The first part of this work concerned the development of complementary methods to screen potential candidates. We used transcriptomic approaches (Gautron et al., 2007) to identify 1) proteins that are specific to magnum, which are responsible for formation of egg white and 2) proteins related to mature shell gland. We also developed a functional screening of antimicrobial proteins using uterine and magnum libraries (Loit et al., 2008). The other strategies were based on biochemical approaches applied essentially to cuticle, egg white and eggshell, all known to play a significant role in the antimicrobial protection of the egg. More precisely, these methods involved identification of candidates by analysis of protein composition of cuticle (the very last barrier against pathogens, which is deposited prior to oviposition) and analysis of egg white fractions potentially enriched in antimicrobial proteins. Results obtained by all these approaches allow for establishing a list of potential antibacterial molecules.

In the second part of this work, we focused on some candidate proteins that we obtained either after purification from egg, after chemical synthesis or after expression as recombinant proteins, and assessed their antimicrobial potential.

Large screening methods

Transcriptomic approaches

Egg formation in the oviduct occurs daily by sequential secretion of the various compartments of the egg around the yolk after ovulation. Each part of the oviduct, magnum (egg white), isthmus (eggshell membranes) and uterus (eggshell), has a very specific role in the synthesis of egg components and large change in expression of genes coding proteins of the corresponding egg components are thus to be expected (Gautron et al. 2007a, 2009).

Therefore, we analyzed the differential expression of genes in magnum compared with other segments of the oviduct (white isthmus and uterus, Figure 3 a and b).

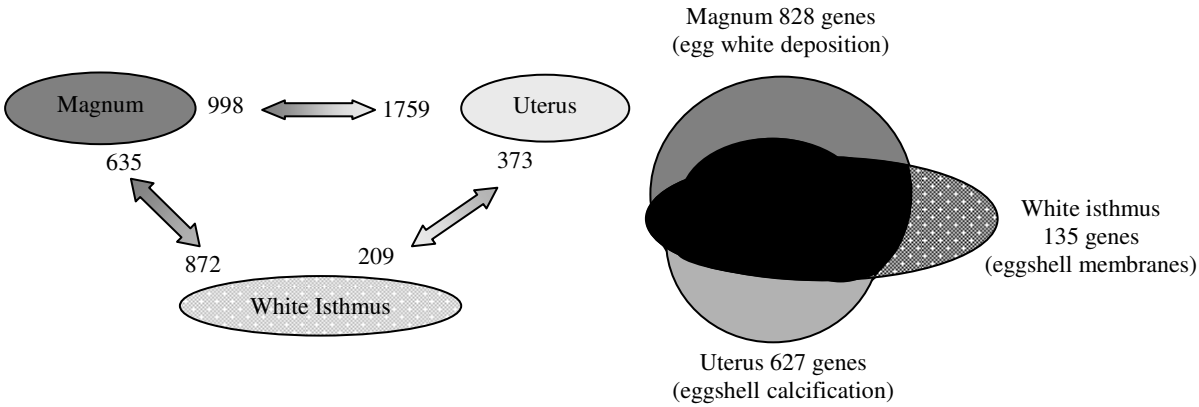

Figure 3a. b.

Using this method, 828 genes were found to be specifically over-expressed in the magnum. Nineteen percent of the specifically expressed genes have no correspondence in databases and might code for novel proteins specific to the hen egg (not expressed in mammalian species). The remaining genes were classified according to their potential antimicrobial activities. *i) Proteins homologous to antimicrobial proteins.* This group includes peptides and proteins containing bactericidal permeability increasing (BPI) domains. The TENP protein, which has high similarity to BPI, was over-expressed in magnum compared to uterus (10 fold) and compared to white isthmus (54 fold). Similarly, beclin-1, which is also related to BPI proteins was over-expressed in magnum. *ii) Proteins involved in antioxidant and inflammatory process, which could be related to host defence*. As an example, a superoxide dismutase, which destroys radicals known to be toxic to biological systems, is over-expressed in uterus compared to magnum (1.57 fold) and white isthmus (1.19 fold). *iii) Genes coding for proteases and protease inhibitors.* Proteases may exert an antimicrobial activity either by degrading bacterial components or by generating antimicrobial peptides by limited proteolysis of other egg proteins. Thirteen peptidases and proteases were found to be over-expressed in magnum compared with the two other tissues. Matrix metalloprotease 23B seemed to be highly specific of magnum (84 fold compared to uterus and about 30 fold compared to white isthmus). Protease inhibitors (antiproteases) could act by inhibiting bacterial proteases that are necessary for proliferation and invasive capacity of pathogens. We found five antiproteases that showed overexpression in magnum including, ovomucoid, ovoinhibitor and ovalbumin related protein X, which belongs to the serine protease inhibitor family, the inhibitory activity of which has never been demonstrated.

In parallel, transcriptome profile of the shell gland have been obtained in sexually immature juvenile and in sexually mature laying hens using a custom made 7K bone and shell gland cDNA array. This study was conducted to investigate genes that are related to active shell gland corresponding to proteins that play a major role for the formation of functional mature eggshell. Genes known to be antimicrobial such as lysozyme G-like 1 or 2 (LYG1, LYG2) were present in the list. An obvious candidate not previously observed was Bactericidal/permeability-increasing protein-like 2 (BPIL2), which was confirmed to be up regulated using RT-PCR with an increase in expression in mature SG of 74145 fold. Secernin1 (SCRN), which has peptidolytic activity was confirmed to be up regulated using RT-PCR with an increase in expression in mature shell gland of 24 fold. Another group, which are less obvious as they were down regulated in the mature shell gland were cathepsin Z (CTSZ), a protease, which was confirmed to be down regulated using RT-PCR by 3.2 fold and ISG12-2 protein (ISG12-2), an interferon-inducible genes, which was confirmed to be down regulated using RT-PCR in mature shell gland by 111 fold.

Functional screening

A high throughput method has been developed, which adapts trypan blue (utilized for discriminating between and enumerating living and dead cells) to screen for novel biocidal molecules emerging from genomes, transcriptomes, or synthetic DNA libraries (Loit et al, 2008; figure 4).

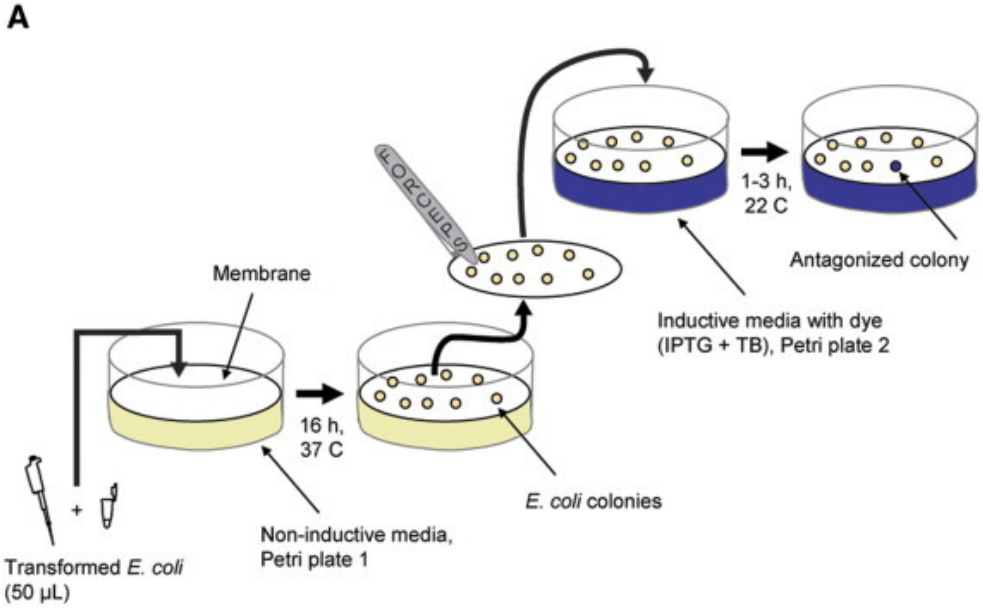

Figure 4. Trypan blue inclusion method.

This approach was used to evaluate a hen uterine cDNA library for identification of cloned sequences that inhibited bacterial growth when expressed. One hundred and twenty plates were screened (about 60,000 colonies), yielding 86 positive (blue) colonies, of which 63 were identified as true positives by secondary screening. The inserts in positive clones were sequenced. Bioinformatic assessment and annotation revealed that the normal role of some of these sequences is related to mitochondrial function (DNAJ (Hsp40) homolog; NADH dehydrogenase subunit 6) or protein synthesis (Ribosomal subunits: S4, S13, L10, L26). Other sequences coded for eggshell-specific matrix proteins (OCX-36, OCX-25, OC-116). The mechanism by which expression of these sequences destabilizes the bacterial membrane leading to trypan blue uptake and growth inhibition is not clear, and may be different in every case. Other inserts did not correspond to any known cDNA, and may code for novel biocidal peptides that are not normally expressed in the chicken genome. Same approach was used to screen a cDNA magnum library. Out of the 6500 colonies that were screened, 96 positive (blue) colonies were identified. All 96 clones exhibited bacterial activity in a secondary screening. The reduction in growth by expressed peptides/proteins varied from 10% to 80%, compared to a control. Twenty eight percent of all initially identified clones (27 clones out 96) inhibited bacterial growth by 50% or more. Only six clones inhibited the growth by less than 20%. Next step will be the sequencing of the clones to identify proteins and analyze the protein sequence to predict their potential function as antimicrobial agents (using bioinformatics tools and homology with known proteins). This work will be pursued in a national french project, Ovomining (2009-2012).

Screening constituents of cuticle

The cuticle of the eggshell may contribute to the eggs chemical antimicrobial defense but because of its insoluble nature, little is known about its protein constituents. Efforts were first conducted to solubilize proteins of the cuticle and minimize contaminants such as keratins, ovalbumin and eggshell proteins. The method of extraction had to be consistent with proper electrophoretic separation by SDS-PAGE for further analysis by Nanoflow HPLC electrospray tandem mass spectrometry to allow the protein identification. We found that the treatment of eggs with EDTA and HCl (1N) allowed for the highest protein yield but the lower protein separation by SDS-PAGE. Treatment with 8M Urea allowed for better SDS-PAGE profile. Mass spectrometry analysis of proteins extracted using EDTA and 1 M EDTA revealed the presence of Ovocalyxin 32 (OCX-32), Ovocalyxin 36 (OCX-36), Ovocleidin-116 (OC-116) and Ovocleidin -17 (OC-17), Ovocalyxin 25 (OCX-25), and Clusterin that all have been described as being components of the outer eggshell matrix suggesting a possible contamination of the "cuticle sample" by eggshell proteins. The 8 M urea extraction method indicated the presence of ovotransferrin, OCX-32, OCX-25, C-type lysozyme, TENP, ovalbumin, ovomucoid and OC116. From these experiments we cannot conclude whether these proteins are specifically expressed by uterus at the time of cuticle deposition or are non-specific contaminants resulting from other egg compartments. However they all appear as potential antimicrobial proteins as part of the cuticle composition. Ovotransferrin, ovomucoid and TENP have known antibacterial activity. The contribution of all other proteins in this antimicrobial potential has to be confirmed.

Screening fractions of egg white

Egg white is known to possess numerous antimicrobial proteins such as ovotransferrin, lysozyme and antiproteases. It is thought to contain many other candidate molecules for which antimicrobial activity has not been characterized yet. In that context, egg white has been fractionated by anion-exchange chromatography to obtain 14 fractions. These fractions were tested for their antimicrobial properties against *Salmonella enterica* serovar Enteritidis and *Bacillus cereus*, gram-negative and -positive bacteria, respectively. None of the fractions tested was found to be significantly active against *Salmonella enterica* serovar Enteritidis in our conditions in agreement with observations of Baron et al., 1997. In contrast, *Bacillus cereus* was very sensitive to egg white and to four out of the 14 fractions obtained. Mass spectrometry analysis of the most active fractions revealed the presence of ovotransferrin, ovalbumin-related protein X and Y, ovalbumin, ovoinhibitor and ovomucoid. Ovotransferrin was present in all fractions analyzed. One of the fractions that showed 100% inhibitory activity against *Bacillus cereus* contained only ovotransferrin as assessed by mass spectrometry analysis. Ovotransferrin possesses distinct mechanisms of bacteriostatic action: one based on the chelation of iron that creates a deprived-iron environment for bacteria, an other based on damage to biological functions of bacterial cytoplasmic membrane (Aiguilera *et al.,*. 2003). In addition, Ibrahim *et al.* (2000) showed a strong bactericidal activity of OTAP-92 a cationic peptidic fragment obtained from hydrolysis of ovotransferrin against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*). These data suggest that ovotransferrin contributes at least partly or completely to the overall anti-*Bacillus* potential of egg white. However the presence of ovalbumin-related protein X (OVAX), ovalbumin-related protein Y, ovoinhibitor or ovomucoid in three of these four fractions suggest that antiproteases might also participate to the anti-bacillus properties of egg white.

In parallel, we used an affinity chromatography to obtain an egg white fraction potentially enriched in antimicrobial proteins. Proteins having affinity for this chromatography would potentially have affinity for bacterial lipopolysaccharide and peptidoglycan and be

antimicrobial. Mass spectrometry analysis of this fraction identified one single major protein recovered in this heparin-binding fraction. Other potential antibacterial proteins were also identified including TENP, avian beta-defensin 11, gallin, avidin, and an antiprotease named "similar to MGC82112 protein".

Assessing antimicrobial activity of purified, synthesized or recombinant proteins and peptides

To verify the antimicrobial activity prediction of all the proteins candidates resulting from large screening methods, one pre-requisite is either the purification of candidates from biological sources, either the chemical synthesis of peptides or the production of the proteins using a heterologous expression system.

Matrix metalloprotease 2 (MMP-2) was first shown using gelatin zymography, a method that reveals gelatinolytic activities. Its proteolytic activity in egg white was shown to vary in function of time and conditions of storage. Interestingly we showed a decrease in its activity in fertilized and non-fertilized eggs incubated at 37.8°C. This protein was further purified by gelatin-chromatography but unfortunately, the quantity of protein recovered after purification was to low to assess its antimicrobial activity. However, regarding its high homology with mammalian MMP-2, egg MMP-2 is more likely to participate in tissue remodeling accompanying embryonic development.

Egg white *ovalbumin-related protein X* was recovered using various chromatography steps, affinity-chromatographies and gel filtration. Although this protein belongs to the serine protease inhibitor family, it does not appear to be an inhibitor of the serine proteases trypsin and chymotrypsin. In contrast to ovalbumin that shares high sequence similarity with OVAX, it was found to be antibacterial against *Listeria* but displayed no activity against *Salmonella enterica* serovar Enteritidis. The analysis of its secondary structures (Circular Dichroism) and tertiary structure (Crystallography) will be performed to be compared to that of ovalbumin. We hope that the resulting data will give information on the structural features distinguishing OVAX and ovalbumin, which could explain their diverging antimicrobial properties.

Avian-beta defensin 11 (AvBD11) was purified from vitelline membrane using heparin-chromatography followed by reverse-phase chromatography. This antimicrobial peptide belongs to the avian beta defensin family the members of which have been described to be antimicrobial. Purified AvBD11 showed antibacterial activities against gram-positive bacteria like *Listeria monocytogenes*, and *Staphylococcus aureus*, but also against gram-negative bacteria, such as *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (Figure 6)

Figure 6. Antimicrobial activity of avian beta-defensin 11 against E. coli (Lehrer et al., 1991),

Gallin, a potential antimicrobial peptide was first identified from egg white after heparin-sepharose and reverse-phase chromatography. Unfortunately it was co-eluted with others contaminants and its concentrations in egg white led to insufficient quantity for antimicrobial and structural analyses. Its length and amino-acid composition was however compatible with a chemical synthesis. The resulting peptide was oxidized to recover a folded peptide containing three disulfide bonds. Preliminary results revealed that gallin had no

effect against *Listeria monocytogenes* and *Salmonella enterica* serovar Enteritidis. We plan to assess its activity against *E. coli* since recombinant gallin has been described as an antimicrobial peptide against this pathogen (Gong et al. (partner 11) submitted). The tertiary structure of synthetic gallin is about to be resolved by Magnetic Nuclear Resonance.

Ovocalyxin 32 is a 32 kDa eggshell-matrix protein, which we originally cloned and found to be abundant in the outer region of the eggshell. It possesses 30% identity with the mammalian carboxypeptidase inhibitor, latexin. In order to further study its function, the recombinant protein was expressed in *E.coli*. Recombinant protein was shown to significantly inhibit bovine carboxypeptidase and also inhibited the growth of *Bacillus subtilis*.

Ovocalyxin 36 (OCX-36; Gautron et al. 2007b) is a 36 kDa eggshell-matrix protein, the sequence of which is homologous to lipopolysaccharide binding and bactericidal permeability-increasing proteins. N-terminal fragment, C-terminal fragment and full length OCX-36 were expressed in *E. coli*. Next step will be the analysis of their antimicrobial potential.

Additionally, we developed an affinity chromatography (trypsin-sepharose) to specifically target active antiproteases from egg white. This method allowed for the purification of *ovoinhibitor* that will be shortly analyzed for its antibacterial activity.

The approaches developed in this workpackage were all complementary and allowed the establishment of a list of candidate proteins and associated genes that were all analyzed for their genetic variation and polymorphism as described in workpackage 3.

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Identification of phenotypic and genotypic variation controlling the antimicrobial defence of the egg.

Introduction

This third work package aimed to identify key factors which determine the variability of the egg defence against bacterial growth and the entry of bacteria to the egg contents. Variation is a feature of all biological systems and the egg is no different. Some of the variation in an egg we can see, for example shape, colour and texture. Other variation is not apparent and we need special measurements to characterise the variation and this is true of bacterial growth and penetration. The variability has it origin in 2 principle components; 1) the environment such as the storage temperature and 2) the genetics of the bird. Both these factors determine how well an egg will prevent bacterial growth and penetration.

In terms of environmental factors we have examined how storage temperature and time interact to alter the antimicrobial activity of egg white. In other words how effective egg white is at preventing salmonella growth, since salmonella is the major concern of consumers and producers. Our efforts to understand the genetic control also looked at how salmonella growth was influenced by the possession of different forms of genes for proteins known to be present in the egg white. We also assessed markers of variation in genes determining eggshell strength which had previously shown promise for selection. Further work was performed to understand how very small cracks, beyond the sensitivity of crack detectors and the vitelline membrane around the egg yolk might contribute to variability.

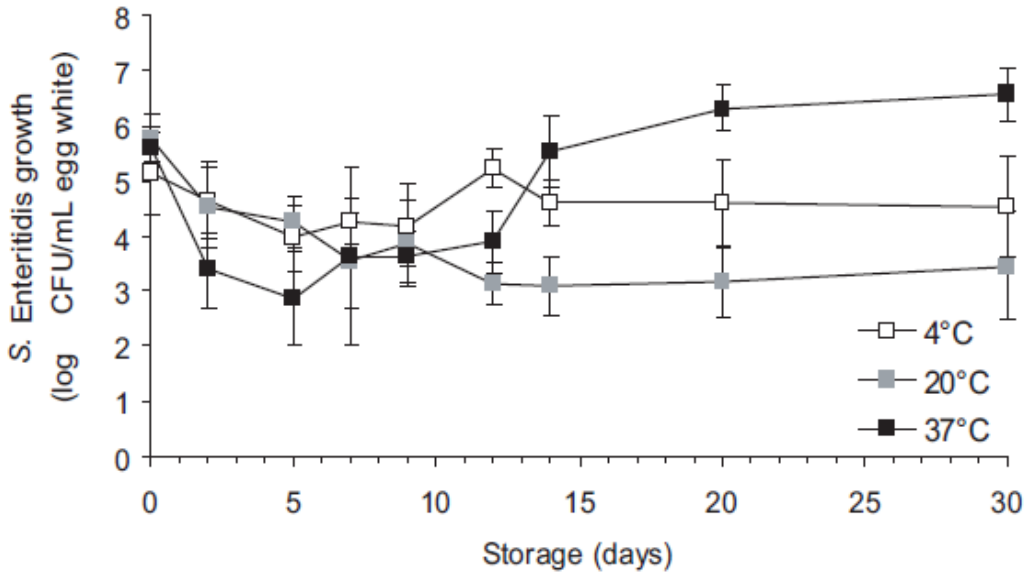
The specific areas addressed were (1) To identify the environmental factors affecting both the hen and the stored egg, which contribute to variation in the eggs antimicrobial properties, (2) To quantify the role of egg structures in contributing to variation in the egg's anti-microbial defence, (3) To determine variation in candidate genes for the egg's natural defence, (4) To determine if the variation in candidate genes accounts for the phenotypic variation observed in anti-microbial activity in a commercial pedigree population and (5) To assess if the markers of genetic variation are suitable for selection to improve the egg's anti-microbial defence. The work was carried out by INRA (France), the University of Glasgow (UK), the Institute for Agriculture and Fisheries Research (Belgium) and the Roslin Institute (UK).

Identification of environmental factors which control variation in anti-microbial activities of proteins in eggs.

Hen egg white contains numerous molecules of interest for human health as they limit toxi-infections (such as Salmonellosis). Little information is available on its antimicrobial potential during storage. Therefore, we analyzed the activity of egg whites against *Salmonella* Enteritidis after storage at 4°C, 20°C or 37°C during 30 days prior to inoculation.
Micro-assays for *evaluation of the antisalmonellic activity of egg white* have been developed for the analysis of the antibacterial potential of a large number of samples (Baron *et al.*, 2006). The microassay methods developed to monitor antimicrobial activity were then used to measure the effect of temperature and storage *on* the antimicrobial potential of egg white against *Salmonella* Enteritidis. In addition, specific assays for the activity of antimicrobial proteins were performed to monitor which activities varied with storage and temperature. It corresponded to assessment of antitrypsic activity, antichymotrypsic activity and antipapainic activity. An important consideration for hatching eggs is the interaction of fertilization and subsequent embryo growth on eggs antimicrobial potential against *Salmonella* Enteritidis.

This was also measured and compared with the profile of unfertilised eggs which are of course used for human consumption.

Regarding the effect of storage, we showed that egg whites displayed higher anti-*Salmonella* activity after a few days of storage at 37°C and that the rate of increase was initially temperature dependent, being higher at high temperature. Egg whites stored at 20°C retained higher antimicrobial activity compared to those stored at 4°C or 37°C when considering the whole storage period. In contrast, storage of egg at 37°C for more than 14 days reduced bacteriostatic potential of egg whites. This decrease was found to be associated with protein degradation and is likely related to pH that varies depending on time and temperature of storage. However no specific correlation was found between the variation of the antimicrobial potential of egg white and that of the protéolytic or antiprotease activities.



.Figure 7. Effect of temperature and storage on the bacteriostatic activity of egg white against *Salmonella enterica* Serovar Enteritidis. Egg whites were independently homogenized and frozen before analysis of their antimicrobial potential. cfu, colony forming units. Initial inoculums, ~10^3 cells/mL of egg white. Results are expressed as mean ± SE.

Interestingly, we observed that fertilized egg whites displayed a lower antimicrobial potential against *Salmonella enterica* Enteritidis compared to egg whites from unfertilized egg whites after four days of incubation. No correlation with protein degradation, proteases or antiproteases activities was identified.

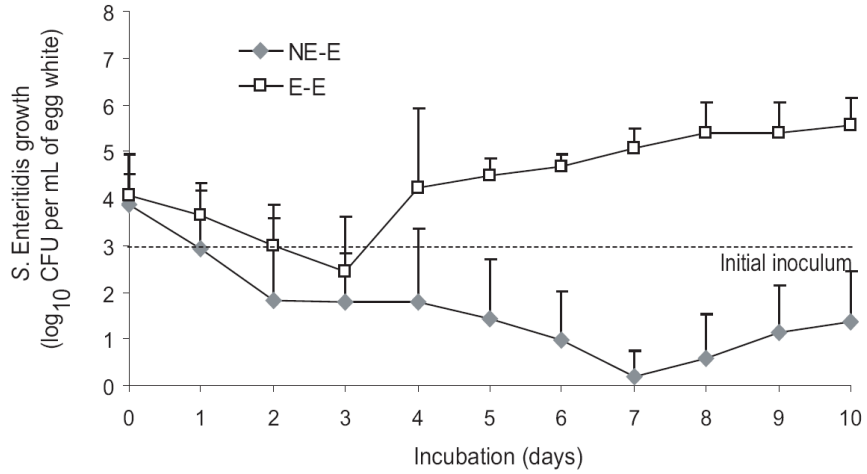


Figure 8: Effect of embryonic development on the bacteriostatic activity of egg white against Salmonella enterica Serovar Enteritidis. cfu, colony forming units. ---- Initial inoculums ~10^3 cells/ml of egg white. Results are expressed as means ± SE. NE=E, no embryo; E-E Embryo

In parallel, we succeeded in the development of ELISA assays for quantification of the antimicrobial ovalbumin-related protein X in egg white.

Identification of traits that control variation in microbial penetration through the eggshell and vitelline membrane.

Role of micro-cracks and the cuticle in bacterial penetration:

Micro-cracks are small cracks which are not observable by normal candling or by the various machines which are used to detect cracks in eggs and indeed are invisible to the naked eye. However, they are a potential route for bacteria to pass from the outside of an egg into the contents and with care they can be visualised once moisture enters the crack (Perianu *et al*, 2007). In this task the hypothesis that microscopic cracks are a major route of bacterial ingress into the egg was carefully tested using controlled induction of micro-cracks. Also the influence of the cuticle was investigated. Micro-cracks were induced in controlled conditions in specific regions of the eggs (four areas *with* and four *without micro-crack*). Then, the density of the cuticle layer was assessed by a dying method (Board and Halls, 1973). The cuticle was removed from the eggs that had few or almost no cuticle (*cuticle absent*); the eggs with a dense cuticle were used as the reference group (*cuticle present*). Following, a comparison was made between areas with and without micro-cracks and between eggs with and without cuticle to determine if they were a significant risk factor that contributes to variation in an eggs resistance to microbial penetration.

Irrespective of cuticle presence and micro-cracks presence, the amount of sites that were penetrated by *Salmonella enteric* serovar Enteritidis (SE) at day 3, 4, 6 and 13 of storage after inoculation onwards was respectively 8 (or 2.00%), 15 (3.75%), 21 (5.25%) and 21 (5.25%) on a total of 400 sites assessed. A trend was observed for an increased penetration in the *presence of micro-cracks* ($p = 0.0546$). For twelve eggs of the 50 eggs studied, penetration was observed on at least one of the eight sites. The number of eggs with penetration on at least one of the sites is higher in the *absence of cuticle* (7/24 or 29.17%)

than in the *presence of cuticle* (3/26 or 11.54%). However statistical significance could not be shown (p = 0.1144).

In conclusion, there is strong indication that micro-cracks are potential routes for bacteria to enter the egg and that the cuticle appears to reduce this penetration. Because the number of penetrations observed was low in comparison to the number of tests made, the power was lower than expected, however the experiments show great promise.

Role of vitelline membrane quality in bacterial penetration

The vitelline membrane (VM) surrounds the yolk. Once organisms such as *Salmonella* pass this barrier there is a massive increase in their growth rate (Gast and Holt, 2000). The hypothesis that vitelline membrane properties were correlated with the occurrence of *Salmonella* penetration was tested using mechanical tests of the strength of the membrane which were correlated with microbiological tests of actual penetration in an *in vitro* test system. Both of these measurements were compared to the quantity and variety of proteins found in the membrane using proteomic techniques and to the ultrastructural properties using transmission electron microscopy (TEM).

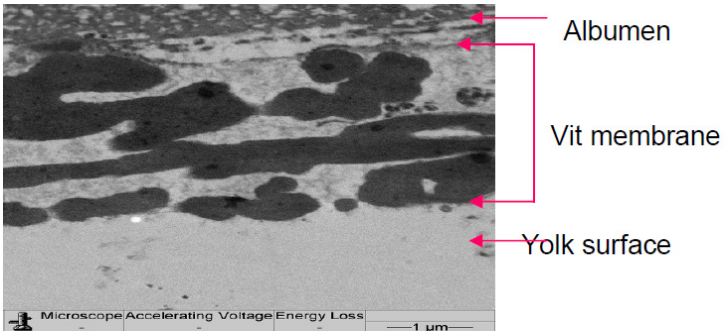


Figure 9 Example of a transmission electron micrograph used for visual scoring of the vitelline (vit) membrane located between the albumen and the yolk.

The mean VM_strength of samples collected at the beginning of lay (0.052 ± 0.0076 N) was significantly higher than these collected at the middle of lay (0.044 ± 0.0062 N) (P < 0.0001). A significant difference between the mean VM_strength of eggs collected at the middle and the end of lay could not be shown. A significant difference between the VM_thickness of samples collected at these laying times could not be shown. The percentage of electron dense material of the VM (VM_% area) of samples collected at the beginning of lay (68.20 ± 5.34%) was significantly (P = 0.0016) higher than at the middle of lay, while no significant difference was detected between the VM_% area of samples collected at the middle and end of lay (54.6 ± 7.7%). The VM_% area is significantly and slightly positively correlated with the VM_strength (r = 0.419; p = 0.011). The proportion of vitelline membrane samples that were penetrated during storage, was hardly affected by the laying period. After one week of storage, 43%, 57% and 55% of the vitelline membranes of eggs collected at the beginning, middle and end of lay respectively, were penetrated. At the end of storage, respectively 9%, 12% and 14% of the vitelline membranes were not penetrated. The VM_time (penetration) was correlated with the amount of an as yet unidentified protein component of the vitelline membrane determined by SDS page electrophoresis and the VM_strength with the VM_% area.

In conclusion, this study displays a slight (r = 0.1751) but significant (p = 0.0086) positive correlation between the VM_time (penetration) and the VM_strength of fresh eggs thus suggesting that a higher VM_strength of a fresh egg leads to a longer resistance of the vitelline membrane against penetration by SE.

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Identification of loci that control genetic variation in microbial defence mechanisms of the egg.

The previous tasks in this work package have dealt with either the sources of environmental variation or are defining new measurements which can be used to define traits or phenotypes that are related to the antimicrobial properties of eggs. Currently, we are looking at the genetic variation in antimicrobial activity. This is the variation which is determined by the inherited material or genes which a hen receives from its parents. To understand the genetic component of variation there are a number values that we need to establish. The individual measurements for eggs from identified hens, known as the phenotype, and the genetic pedigree of these hens, this is the subject of this task. We also need to be able to assay the variation in the genetic material that exists in the population that we have phenotype for and that is known as the genotype. Two new populations with phenotype and genomic DNA were produced. Population 1 (n= 1700 hens) with phenotype for salmonella growth in egg white for 2 eggs and population 2 (n=1300 hens) with phenotype, breaking strength, Kdyn, deformation, total thickness, effective and mammillary thickness for 2 eggs.

The second step was to test candidate genes alleles for association with phenotypic traits affecting the risk of microbial contamination. Fifteen gene loci chosen from the literature or from studies with in other work packages (Table 3.1) were examined for polymorphism in the target population. Not all the gene loci produced useable polymorphism.

Table 3.1

Gene	**Function**
Cystatin	Cystatin is a sulphydryl proteinase inhibitor (Saxena and Tayyab, 1997)
Ovostatin	Inhibits metalloproteinases (Nagase and Harris, 1983)
Avian β-Defensin11	Cationic membrane disruptor
Avidin	Binds biotin (Elo et al., 1980)
LysozymeC	Lysozyme has bacteriocidal muramidase activity that hydrolyses glycosylated proteins in bacterial cell walls, particularly gram positive bacteria, but there are other bacteriocidal domains with independent function (Pellegrini et al., 1997)
Ovotransferrin	The anti microbial action of ovotransferrin is attributed to both structurally dependent bacteriocidal effects and bacteriostatic iron chelation (Ibrahim et al., 1998)
Tenp	Tenp, a protein with strong homology with a bacterial permeability-increasing protein family (BPI), (Guerin-Dubiard et al., 2006)
Ovomucin	Ovomucoid has trypsin inhibitory activity and may inhibit serine proteases (Odum, 1987)
Ovoinhibitor	ovoinhibitor inhibits bacterial serine proteinase, fungal serine proteinase and chymotrypsin (Saxena and Tayyab, 1997)
Gallin#1	Cationic membrane disruptor (Gong et al., 2009) (From WP2)
Gallin#2	Cationic membrane disruptor (Gong et al., 2009)
Gallin#3	Cationic membrane disruptor (Gong et al., 2009)
Hep21	Egg white protein with binding properties to bacterial LPS. A new member of the uPAR/CD59/Ly-6/ snake neurotoxin superfamily. (Nau et al., 2003)
Lipocalin	Roles as siderophores (Flo et al., 2004) (From WP2)

Thirty six variants from these gene loci were genotyped in the entire population for salmonella growth in egg white along with 5 markers, ovocalyxin 32, oestrogen receptor, ovocleidin 116_310, ovocleidin 116_1336, carbonic anhydrase_1210, ovalbumin in the population for shell quality. Test for association completed between the markers and the traits using a regression analysis.

Thirdly we identified loci that control genetic variation in microbial defence mechanisms of the egg. The eggs defined proteome makes determining the loci which contribute to the genetic variation in antimicrobial defence an attractive proposition. The presence of large number of differences in the DNA between hens in pedigree populations was confirmed in this study with 119 single nucleotide polymorphisms (SNPs) being observed. Of these 77% were novel and 20 % altered the amino acid sequence of the protein the gene coded for. This latter figure is somewhat inflated by the targeting of coding exons in the SNP discovery exercise. What was perhaps surprising was the low level of variation observed in some of the genes, notably in all 3 forms of Gallin, lipocalin and in avian beta defensin 11. The reason that we were surprised is that we had observed the rate of variation in many antimicrobial genes was greater than that observed in other genes and, at least in the case of gallin, we had evidence that there was considerable variation in lines of hen not related to the pedigree lines being studied. This suggests that selection pressure may have been quite strong in laying hens to fix the alleles. Unfortunately we can not answer the question of whether these forms are more advantageous in reducing bacterial growth, since we could find no genetic variation in the lines that we have tested for antimicrobial activity.

We were also able to confirm in this study that salmonella growth in egg white was a moderately heritable trait with an estimate of 0.23. This suggests that if the assay could be made more accessible and possibly more reliable it could even be used directly.
When we tested for association between the markers for genetic variation in the antimicrobial genes and salmonella growth we observed alleles of lysozyme and ovostatin that may contribute to variation in salmonella growth in egg white (Table 3.2).

lysozyme_e01_246	GG	GA	AA
P=0.04	**1.51**	**1.64**	**1.54**
Size of effect 0.11	13% of trait SD		
lysozyme_e02_061	TT	TC	CC
P=0.006	**1.50**	**1.65**	**1.54**
Size of effect 0.13	15% of trait SD		
GG_ovosta_e25_417	GG	GA	AA
P=0.006	**1.64**	**1.56**	**1.49**
Size of effect 0.07	8.5% of trait SD		

Table 3.2. The featured markers have effects of the size we have typically come to expect of such associations. It should be remembered that the figures represented in the size of effect are log cfu. and therefore represent relatively large changes in salmonella growth.

In the replication experiment for association between the markers for genetic variation in the egg shell genes and egg shell quality we were also able to confirm that ovocleidin 116 as an important contributor to variation in eggshell thickness, an important trait in preventing contamination of egg contents.

All of these markers are now available to use or to test further by the breeding industry.

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Based on their characteristics, some eggs might be more subject to bacterial contamination than others. RESCAPE project, therefore, aims in this section at improving the non-invasive technology of measuring egg quality used for egg grading as a way to enhance the detection level of risky eggs and hence reduce the potential risks of eggs bacterial contamination. Moreover, this technology will become available for project partners in an easy to use measurement package. This work was carried out by the Catholic University of Leuven.

Detection of cracks in eggshells

Nowadays, hairline cracks in shells are detected using vibration techniques (e.g. Acoustic Egg Tester) with high accuracy and at a high rate (Coucke et al., 1999 and De Ketelaere et al., 2000). However, recent research has shown the presence of micro-cracks in eggshells (Bain et al., 2006). The dimensions of these micro-cracks are smaller (less than 5 mm in length) compared with the common known hairline cracks. Such micro-cracks do not influence the mechanical behaviour of eggshells and hence are invisible for modern crack detectors. Being a potential entry for micro-organisms, the study of these micro-cracks is highly relevant.

Figure 10. Acoustic Egg Tester

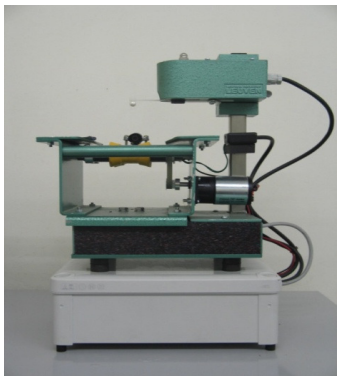
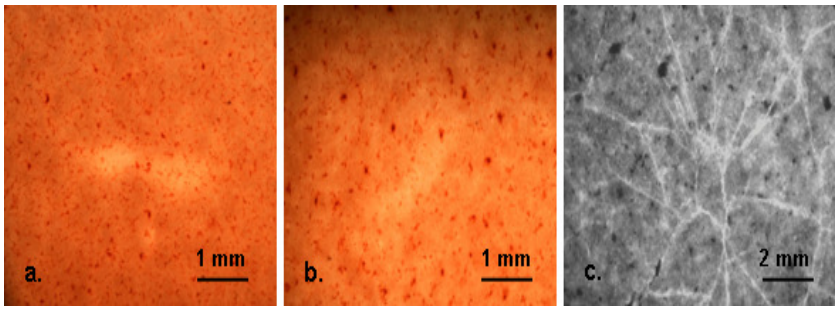


Figure 11. Types of failure in eggshells



11.a Naturally formed micro-crack
11.b Artificially induced micro-crack
11.c Hairline cracks network at macroscopic failure

A first experiment concerns the parameters affecting the micro-cracks creation, in relation to the general behaviour of damage in eggshells. The eggs used in this study originate from Isa Brown® hens housed in battery cages. Typically, eggs were stored for maximal two days at the start of the experiment. Detection of such micro-cracks was done visually after 24 hours of crack generation such that albumen liquid entered the micro-crack making it visible. In a second experiment, Acoustic Emission (AE) techniques are used during compression in order to determine the load needed for micro-cracking for each individual egg. During the same test, eggs were incrementally loaded until failure (macro-cracking). This way, information about the load needed for micro and macro cracking is available. These measurements are then used to correlate micro and macro crack forces. Since breaking force (macro-cracking) is predictable from acoustic measurements, a high

correlation between micro and macro cracking force can provide a means for detecting risky eggs in terms of micro-cracks.

Firstly, it was found that the average force value required to artificially induce micro-cracks in eggshells is 13.75 N. A time period of around 24 hours is more or less the time needed for the albumen liquid to enter the micro-crack and to make it visible. Eggs with a lower resonant frequency and a lower dynamic stiffness have a higher risk to be micro-cracked. This was already shown for hairline cracks and therefore an important outcome of this research is that the micro-cracks behavior is similar to the one specific to common known hairline cracks.

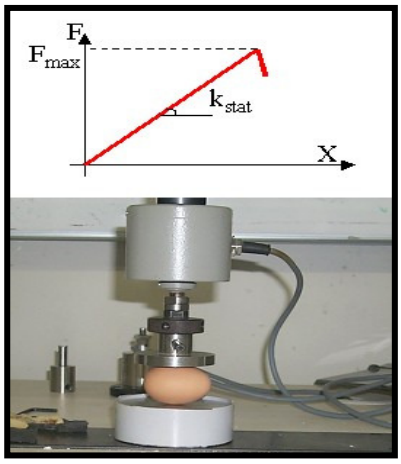


Figure 12. Artificial creation of cracks in a compression loading test

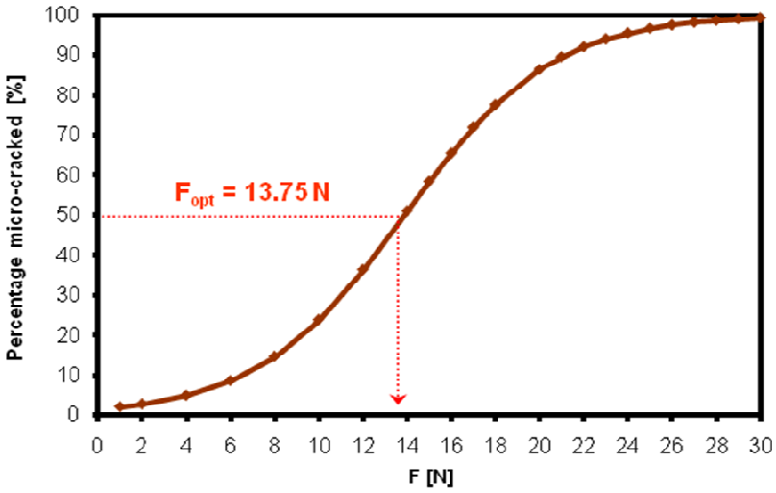


Figure 13. Optimal micro-cracking force

Secondly, micro-cracking of eggs was investigated by means of Acoustic Emission (AE) techniques and laser ultrasonic detection methods. Sensors (accelerometers, force and strain sensors) covering low frequency as well as high frequency range were used.

Figure 14. Acoustic Emission detection

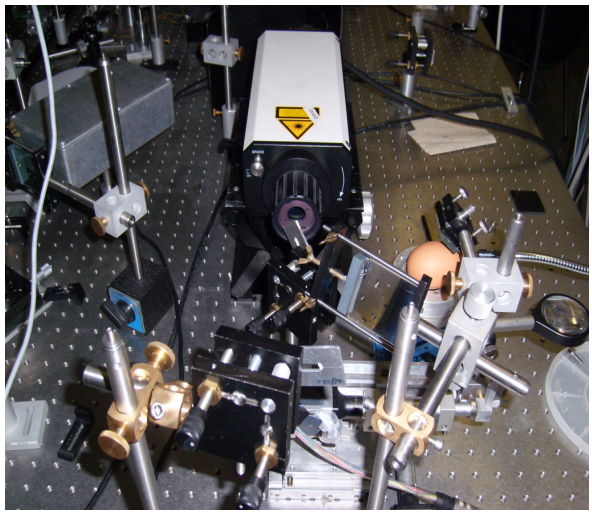


Figure 15. Laser ultrasonic detection

Post-processing of measured signals showed that the acoustic energy released by these microstructural changes is not enough to determine a detection algorithm based on the acoustic emission signatures. Moreover, it was shown that high complexity detection techniques (nonlinear vibration measurements, laser ultrasonic detection) do not apply to microcrack investigation. Further research is needed to investigate the AE activity associated to the phenomenon of microcracking in eggs.

Spectral detection of risky eggs

Another goal of this project was to investigate whether the bacteriostatic and bacteriocidal properties of the albumen can be assessed by means of optical transmission measurements (VIS/NIR method). For this, spectral measurements were performed both on egg albumen and on intact eggs. Subsequently, anti-microbial activity (AMA) was determined on the albumen. Finally, correlations between spectra and AMA were investigated using Partial Least Squares (PLS) techniques in Matlab software (version 2007b, Mathworks®). Firstly, an 'ideal' experiment was conducted in which spectral measurements were performed upon the albumen and the AMA was determined on individual eggs. Secondly, spectral measurements were performed during an ongoing large-scale experiment. Spectra were measured on intact eggs and AMA values were determined on pooled albumen.

Figure 16. Spectral detection set-up

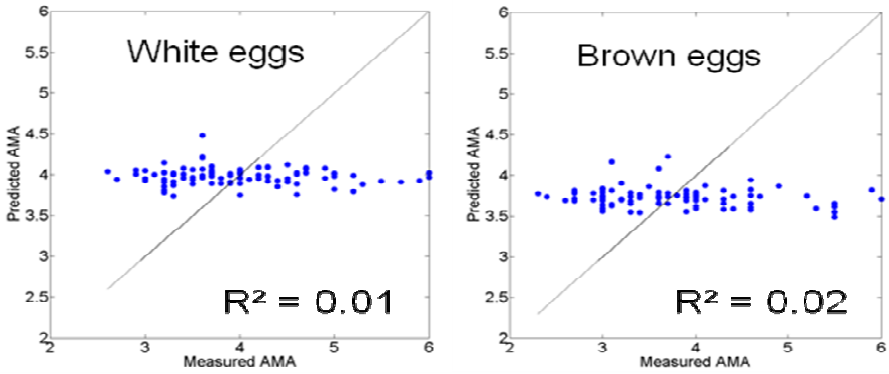


Figure17. Spectral measurements on both albumen and whole egg did not contain information with respect to antimicrobial activity (AMA)

PLS analysis showed that the spectral information obtained from albumen and whole egg does not contain information with regard to anti-microbial activity (AMA). Furthermore, it should be noted that the standard deviation (s.d.) obtained for the AMA when measured repeatedly on the same sample is large (0.54) when compared to the overall standard deviation that was observed between all eggs under investigation (0.77) indicating that the reference measurement is not very accurate.

Dirt detection on eggshells

An off-line computer vision system to differentiate and quantify the presence of different dirt stains on brown eggs has been developed at KULeuven (Mertens et al., 2005) and was therefore the initial proposal for dirt detection on eggs. This technique has some limitations such as the fact that is time consuming (15 s/egg) and not accurate enough to

distinguish between bloodstains and dark stains. As an alternative to this detection method the hyperspectral imaging technique was applied for the evaluation of dirt on eggshells. The aim of this work was to deliver a tool able to respect the industrial criteria for dirt detection on eggs. The major objective of this study was the selection of wavelengths offering the best contrast between dirt and clean eggshells. Several types of dirt can be found on the eggshell but for this initial study 4 types of dirt were investigated: yolk stains, albumen stains, urine and faeces.

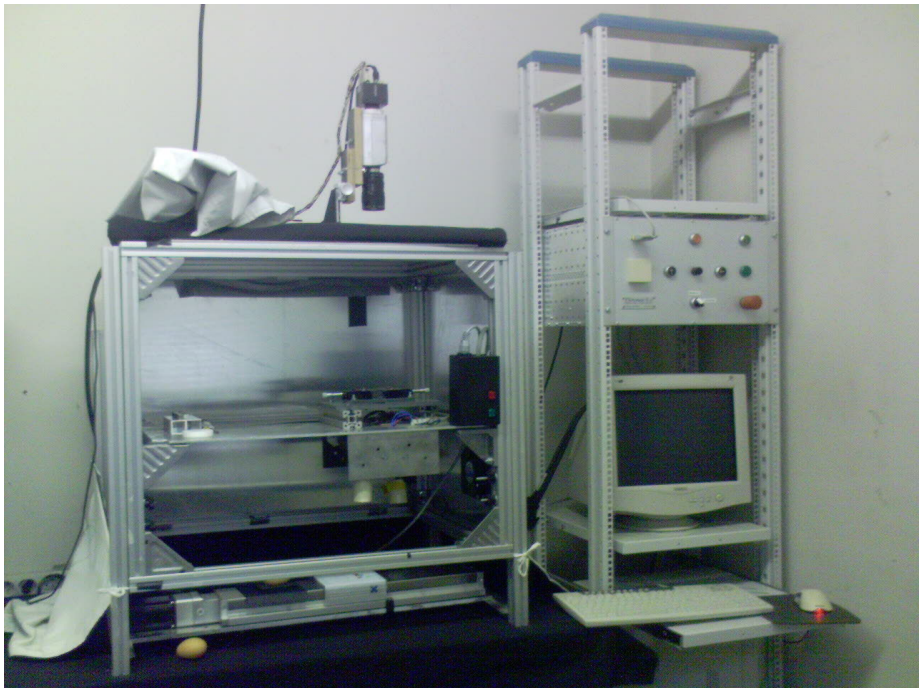


Figure 18. Hyperspectral imaging system

It was shown that 680 nm is the optimal wavelength to detect faeces on eggshells, 480 nm – 550 nm for urine detection, 500 nm for yolk stains and 550 nm for albumen stains. Moreover, natural pigments of the eggshell were detected for a wavelength value of 650 nm. Consequently, when filtering in the near infrared (NIR) region, above 700 nm, and a deviation will be observed, this deviation will certainly be a type of dirt since natural pigments are not visible for this waveband. Furthermore, any deviation detected in this waveband range can be associated to the presence of faeces on the eggshell. However, when there is nothing observable in the NIR region, it cannot be concluded that the egg is clean. The waveband ranges associated to yolk and albumen stains are both present in the detection range of urine. This is an advantage because urine reflects while yolk and albumen absorbs, so only one waveband range is needed. When strong reflection is present between 480 nm and 550 nm, the type of dirt detected is urine. If the absorption at 500 nm is higher than the one at 550 nm, then yolk stains are present. A waveband value of about 500 nm is indeed the optimal waveband for yolk detection. When the absorption at 550 nm is higher than the absorption at 600 nm, then the detected dirt is albumen, if it is not the case then natural pigments of the eggshell were detected.

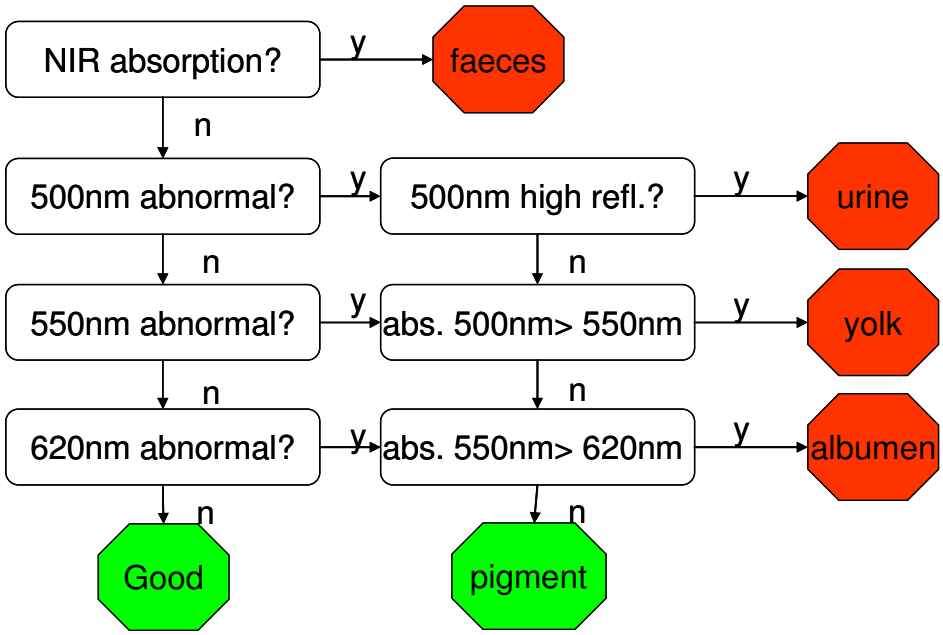


Figure 19. Dirt detection algorithm

Generally, detection of any deviation unrelated to albumen, yolk, faeces or urine, means that natural pigments of the eggshell were measured.

Development of a non invasive quality measurement platform using previous techniques

The global objective of this research was to combine crack detection, spectral detection and dirt detection in one measurement package able to indicate the risk level of eggs from a complete perspective. For the eggshell strength and the crack presence evaluation, the Acoustic Egg Tester was included in the 3 in 1 platform after performing some mechanical and electrical adjustments on the old design. The plastic hammer of the Egg Tester, which is normally mounted above the rollers, was now placed under the rollers not to be in the way when taking images for dirt detection. The spectral detection is based on the classical optical set-up, slightly adapted to the actual design. The light source was in this case a halogen lamp with an flexible optical fiber guiding the light to the egg. Finally, the dirt detection operation required a completely new design based on the hyperspectral imaging concept. The measurement platform is controlled by a fully new software. Part of the code was already written and was therefore just adapted in order to be included in the final software. The global code was created using the LabVIEW program, a graphical programming environment developed by National Instruments.

A non-destructive measurement platform combining crack, spectral and dirt detection is now available. The effective design consisted in a fusion of existing techniques and new measurement concepts in one measurement package. The eggs are checked for cracks and the dynamic stiffness of the eggshell is calculated. The entire egg spectrum is stored and the eggshell colour index (TCV value) is determined. The third operation available on the measurement platform is related to the dirt presence on the eggshell, the dark and white dirt

stains are detected and both dark stains perecentage and white stains percentage are finally displayed.

The final output of the measurement platform consists in a crack presence alarm, an eggshell colour index (consumer criterium and indicator of stress and general bird health) and a dirt percentage. Based on the previous outputs, the final code will be upgraded to be able to give “risky eggs” warnings. The critical thresholds to define “risky eggs” will be determined from a large scale experiment.

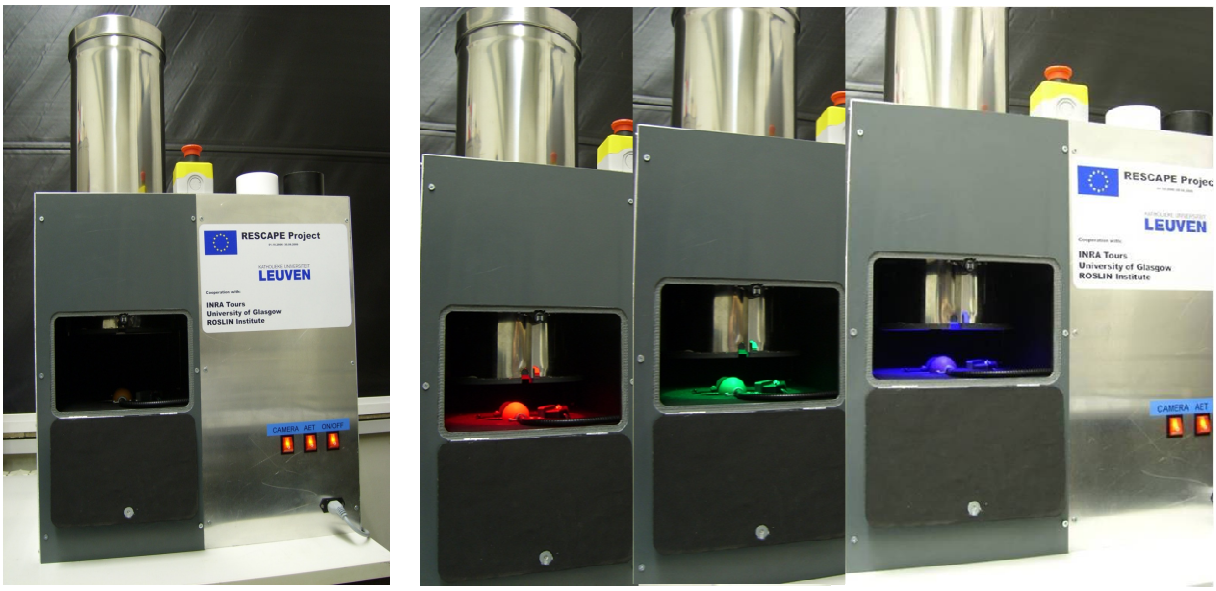


Figure 20. Easy to use prototype of the measurement platform

Chicken flock monitoring system for risks and diseases

The basis for direct detection of risky eggs is the frequent assessment of aspects of egg quality which are related to the protection capacity of the eggs against bacterial attacks. Frequent measurement of quality aspects enable to perform monitoring and signal a loss of quality (increased breakage, decreased shell strength, increased dirtiness, reduced pigmentation indicating stress or disease). In this section, the quality of eggs of several flocks of laying hens was monitored in time in order to detect quality problems.

To investigate this, two monitoring studies were performed. The first study was carried out on the eggs of 10 layer flocks in Belgium which were investigated during the complete laying period, and the second study was performed in a grading plant of GLON (Le Cam) in Naizin, France, where the eggs of 11 commercial layer flocks were tested during 3 months. The eggs in this study were tested with the Acoustic Egg Tester (AET) and the Transmission Spectroscopy. The AET is used to define shell integrity (% Cracked eggs) of the eggs and shell strength of the intact eggs defined as Dynamic Stiffness (Kdyn). The Transmission Spectroscopy setup is used to define the pigmentation of the eggs, defined as the Transmission Colour Value (TCV). The AET was used in each of the 10 layer farms in Belgium and in the grading plant in France. The Transmission Spectroscopy setup was only used in 1 farm in Belgium.

To be able to detect deviating values of the recorded measurements, an Intelligent Monitoring System (IMS) is used. The algorithms that form the basis of the IMS consist of a combination of the concepts of Statistical Process Control (SPC) and Engineering Process Control (EPC). The combined application of these concepts for controlling a production process is called Synergistic Control. In this algorithm EPC strategies are used to pretreat the raw data in order to remove the statistically inconvenient properties of egg quality data. After this treatment the corrected residual data can be introduced into the cusum (cumulative sum) control chart. A control chart SPC aims at discerning between normal or common cause process variation and abnormal or special cause variation and generating alarms in case of abnormal variation as a result of problems. The result of this algorithm is a cusum control chart with alarms for the observations which were evaluated as being out-of-control. As such this control chart is used as a tool for management support of the production process of consumption eggs. For more detailed information on the IMS, see Mertens et al. (2008), Mertens et al. (2009) and Mertens (2009).

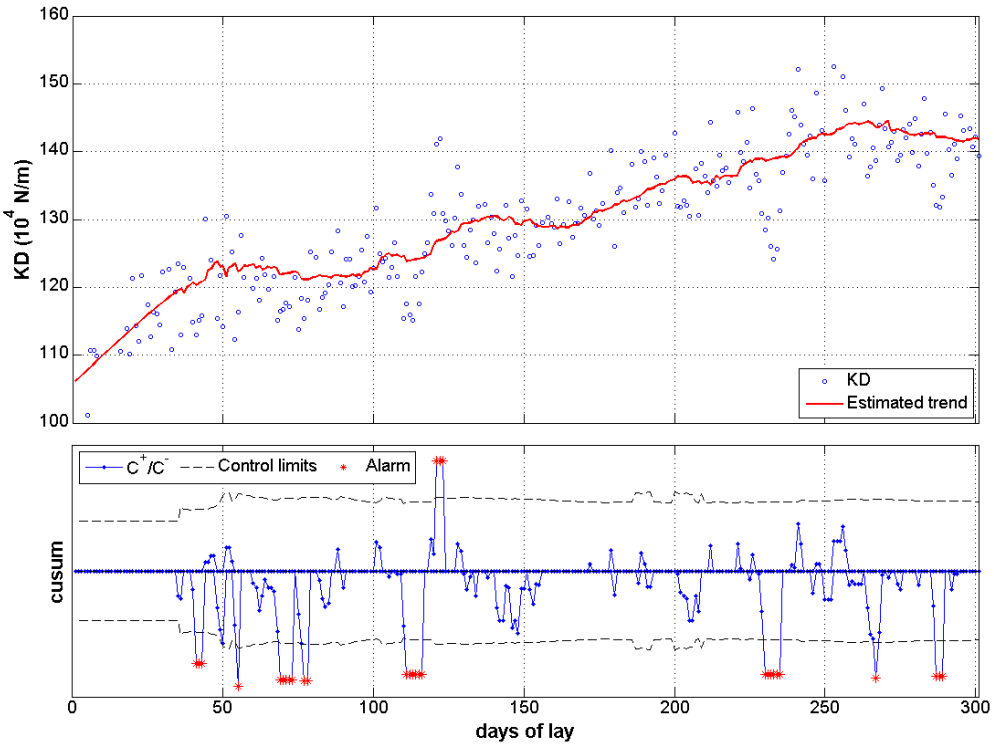


Figure 21. Cusum control chart for the Dynamic Shell Stiffness (KD). Upper graph: raw KD data with mathematical trend detection. Lower graph: Cusum chart with alarm situations indicated by the red asterixes (*).

From the results of the monitoring study it is clear that the separate egg quality measures – shell integrity, shell strength and shell colour – provide valuable information on the protection capacity of eggs. For example, lower shell strength as a result of heat stress was signalled, an Infectious Bronchitis infection of the hens was quickly signalled, and a high amount of cracked eggs was noticed by the monitoring while it remained unnoticed by the sorting plant.

These measures can directly indicate risky eggs – e.g. a cracked egg with dirt on it can be directly indicated as risky – or can be an indirect indication for a higher likelihood of the occurrence of dirty eggs – e.g. when the shell strength (KD) reduces, the likelihood of the weak eggs getting cracked in the rest of the complete logistic chain increases considerably. When these measurements are combined the direct fusion of these separate information flows enables to quickly detect risky eggs or detect problematic situations (disease, stress) before risky eggs are generated.

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Innovative egg treatments for reducing bacterial contamination of egg surface and contents

By 2012, Council Directive 1999/74/EC, defining minimum standards for the welfare of laying hens, will abolish conventional cage systems in favour of enriched cages or alternative systems in order to improve the welfare of hens. However, keeping hens on the floor or outdoor could present an increased risk of bacterial contamination since the total count of aerobic bacteria is generally significantly higher for nest eggs from non-cage systems compared to eggs from enriched or conventional cages even if there is not a common scientific agreement on this subject.
In this regard the introduction of efficient measures to reduce eggshell contamination by S. Enteritidis or other bacterial pathogens, and thus to prevent any potential or additional food safety risk for human health, may be envisaged. In USA egg washing, together with the use of cold storage, is at the present time the most common tools to control bacterial contamination. These procedures are forbidden in Europe for class A eggs (except for The Netherlands and Sweden were egg washing is applied on a voluntary basis) since egg washing is claimed to damage the cuticle of the egg thus favouring the penetration of bacterial pathogens if best practice washing procedures are not used. Therefore alternative treatments should be investigated for the surface bacterial decontamination of table eggs. In this regard two possible approaches may be envisaged: the approach “one shot” and the approach “long-term”. Regarding the approach one shot, the egg is treated “one shot” before the commercialisation. The approach long-term requires the application of techniques able to maintain their decontamination effect all over the chain from farm to fork.
In this workpakage microwave, hot air and gas plasma were studied as one shot treatments along with modified atmosphere packaging and chitosan coating as long-term approaches.

Eggshell decontamination using microwave

The work on this topic was planed as a cooperation between the Catholic University of Leuven and the microwave company MEAC (Belgium) in order to deliver a microwave decontamination treatment for eggs. Eggs are potential hosts and carriers for pathogenic microbes like Salmonella enteritidis, due to their rich nutritive value. Heat pasteurisation is the best solution for controlling these pathogens.

Nowadays, there is no available technology for pasteurising whole shell eggs. The major disadvantage associated with common pasteurisation techniques of food products is the non-uniform temperature distribution. This is one of the greatest challenges in pasteurisation, especially when applying such a heat treatment to shell eggs. In this section, the potential benefits of using microwave energy for heating fresh in-shell eggs to pasteurisation temperatures were investigated. Generally, microwave treatments can be used to raise the temperature of in-shell eggs to the required pasteurisation temperature in minutes. Moreover, it was shown that microwave heating induces thermal destruction of microbes (Tajchakavit, 1997).

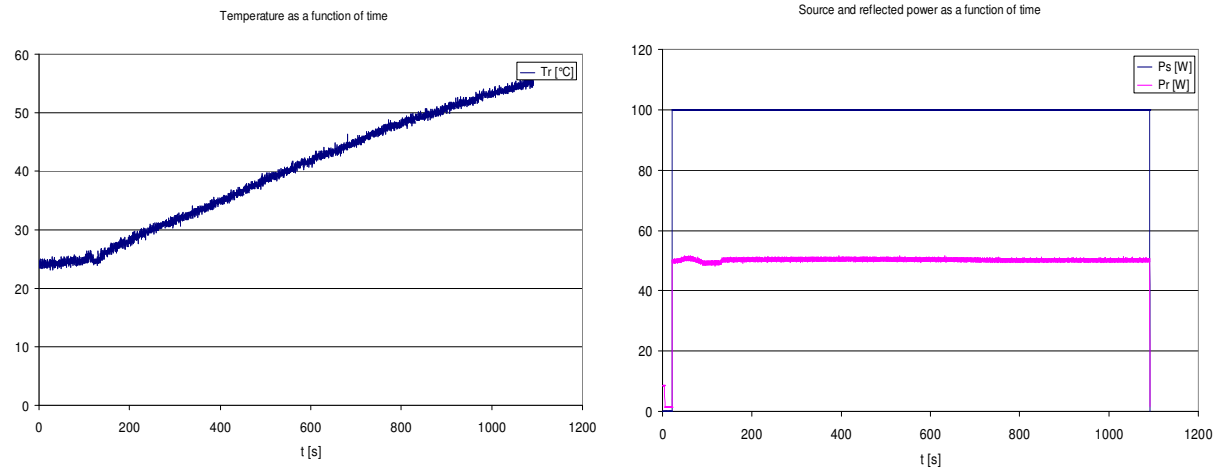
The time needed to pasteurise foodstuffs is function of the applied temperature. This relationship differs for different types of micro organisms. In eggs, the most important micro organisms for public health are Salmonella species. During heating of albumen, proteins in the albumen will change their conformation and the overall albumen quality will change (Donovan et al., 1975). Therefore, a good knowledge of this coagulation temperature as well as a measurement technique for albumen quality should be available.

In the present research, the measurement of turbidity as a reference for albumen quality was used. Turbidity of a fluid found its origin in the evaluation of water quality and can

be calculated after an optical transmission measurement through a sample of the investigated fluid. The advantage of this optical measurement technique is its simplicity, its fixed protocol and therefore it is generally applicable in different kinds of laboratories (Fernandez-Lopez et al., 2006; Van der Plancken et al., 2006; Matsudomi et al, 2004; Vuckovic et al., 2005; Weijers et al, 2006). Moreover, optical equipment becomes cheaper, making this measurement technique available for all quality measurement laboratories.
In this study, two experiments were done. In the first experiment, the evolution of the turbidity in three albumen samples was studied during heating from room temperature towards 63°C. During the heating period, the evolution of temperature and turbidity was registered each time the temperature increased with one degree. In the second experiment, the time/temperature effects on turbidity were studied in the range where turbidity was shown sensitive to heat treatment in the first experiment. Samples of albumen were warmed until a fixed temperature (50, 52, 55 and 58°C) was reached and maintained during 15 minutes at that fixed temperature. For each temperature, 3 samples were heated. During this period of constant temperature, turbidity was measured each 20s, resulting in a total of 45 turbidity measurements. The turbidity study showed that side effects on albumen quality can be expected from 59°C while heating for very short periods (periods smaller as half a minute, first experiment). When heating for longer periods (second experiment), the maximal temperature to prevent coagulation is even lower. Here, for longer heating periods, coagulation effects can be expected already between 52 and 55°C, as reported before in literature (Fernandez-Lopez et al., 2006; Van der Plancken et al., 2006; Matsudomi et al, 2004; Vuckovic et al., 2005; Weijers et al, 2006).

A finite element model combining conduction heat transfer with microwave power dissipation and a first order bacteria inactivation kinetics was built in COMSOL Multiphysics 3.4 (Comsol AB, Sweden). The model distinguishes the individual compartments of the egg, i.e., the eggshell, air chamber, albumen and yolk. The model consists of the Fourier equation for time and space dependent heat transfer. The power dissipation was modelled assuming an exponential decrease of power density inside the material. The input parameters of the model include the thermophysical (Denys et al., 2004) and the dielectric (Dev et al., 2008) properties of the egg components. The boundary condition of the egg heating is the convective heat transfer from the egg shell to the environment during the microwave process. The microbial inactivation model uses first order kinetics of Salmonella Enteritidis. The finite element model considers the actual geometry of the axi-symmetric egg shape and its internal components. On this geometry, a finite element mesh is defined to discretize the heat transfer and lethality model equations in the spatial coordinates. A two-steps pasteurisation process was simulated using the finite element model. The egg was heated in a first step for 100 seconds under electric field E = 1000 V/m (microwave ON, constant at egg shell surface) while in the second step of the treatment, for t > 100 s, the electric field was E = 0 (microwave OFF), the air temperature was 57°C and the air velocity 1 m/s. Microwave heating allows fast heating, then air heating for uniform equilibration. The most important outcome of the computer simulation was that although a very fast heating corresponding to the treatment first step, such a process requires much more time for the bacteria inactivation phase (~ 30 minutes).

Based on the simulation outputs, an experimental microwave treatment was applied on fresh eggs. The temperature measurements obtained for the eggs at a given power level (100W) were plotted as a function of temperature versus time to observe the heating curves within the pasteurization temperatures and also to study the actual heating time required for shell eggs to reach the pasteurization temperatures (Figure 22). The data obtained was used to calculate the come up time (the time taken to reach the pasteurization temperatures) for the input power of 100W. As shown in the power distribution plot, only half of the input power was transformed in microwave energy during the treatment, the other half being reflected. Due to this loss of power by reflection, the overall time to reach pasteurization temperatures during shell heating was found to be about 15 minutes, corresponding in fact to a real power of 50W.



Heating curve of eggs at 100W Power distribution

Figure 22. Experimental microwave treatment

Temperature plots of the egg content were taken using an infrared camera (Figure 23). The visual examination showed no coagulation of the albumen. However, a non-homogeneous temperature distribution should be noticed. The yolk had heated up faster than the albumen. A possible reason for this phenomenon can be the focusing effect of the egg shell curvature. The spherical geometry and the central yolk position inside a shell egg have resulted in a convergence of the microwave energy towards the centre hence increasing heat dissipation in the yolk. In addition, the radial penetration depth and loss/attenuation of the microwave energy could have contributed to the higher heating rate of the yolk.

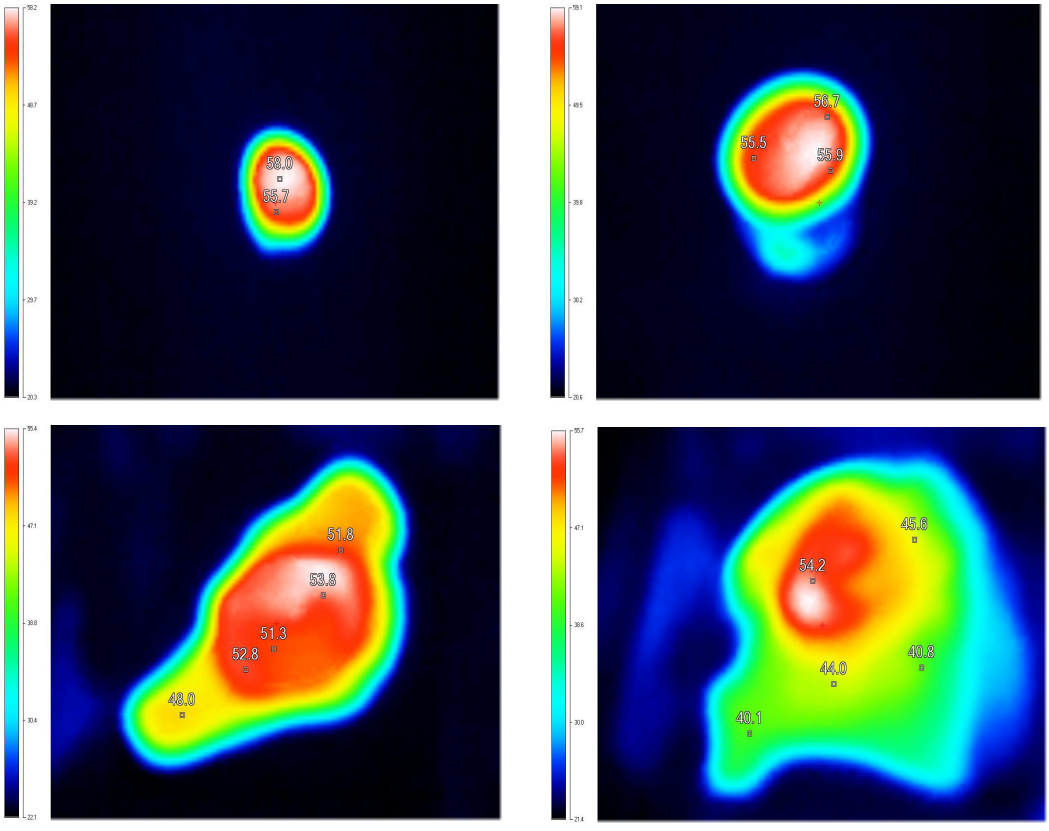


Figure 23. Temperature profiles of the egg content after microwave treatment

When thinking of microwave heating of a shell egg, the first thing that comes to mind is the high risk of great pressure build-up within the eggs. However this is not inevitable within the pasteurization temperatures. With proper control of the process parameters, microwave heating can provide efficient and rapid heating for thermal pasteurization. The issue of microwave heating uniformity can be overcome with the proper orientation of the egg and a specially designed waveguide, which is an engineering issue and also by the precise design of the container taking the eggs into the microwave chamber (Yakovlev, 2001).
Since the industrial partner, the microwave company MEAC, abandoned the project, the planned experimental work sequence (treatment optimisation – side effects – lethality of the treatment) was not accomplished and the initial objectives were only partially fulfilled. At this point, further investigations are needed for designing an appropriate decontamination treatment. A comprehensive assessment of the functional quality of the microwave heated eggs will have then to be done by examining the changes in the physical properties responsible for that (foamability, turbidity etc...). Finally, the effectiveness of the treatment will be assessed by looking at the lethality of the microwave heating on eggs inoculated with reference bacteria like Salmonella Enteritidis.

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Hot air pasteurization

This task was carried out by the University of Bologna. Within this task the efficacy of hot air in decontaminating the surface of shell eggs was investigated together with the possible side effects of the treatment on different quality traits of the egg. To study the problem, initially a simplified numerical model was developed describing the thermal interaction between the air and the egg, using a computational fluid dynamic tool (CFD) based on Finite Element Technique. To validate the CFD model an egg simulant was set up (Fabbri et al., 2007). Tests conducted at different combinations of air flow rate and treatment duration were compared with simulated results. The numerical model, once validated, permitted to quickly evaluate a high number of testing conditions. Particularly it was solved in transient conditions, as a virtual bench, sampling the parameters space: air temperature; air speed; duration of the treatment; revolving speed of the egg and rest interval between successive treatments. On the basis of the experience with the numerical model and the simulant, after some preliminary tests on experimentally inoculated eggs, a particular thermal cycle was selected. A specific apparatus was built to treate the eggs (Figure 5.3).

This was composed by two hot air generators fixed in a definite position by a hard fixture over a roller conveyor. The rolling cylinders are moved by a transmission belt, linked to a stepping motor served by an electronic speed regulator. The cold air flows, about at ambient temperature, from a nozzle in the lower part of the roller conveyor and it is served by a needle valve for flow partialisation. The cold air came from a compression plant, able to maintain a nearly costant feeding pressure.

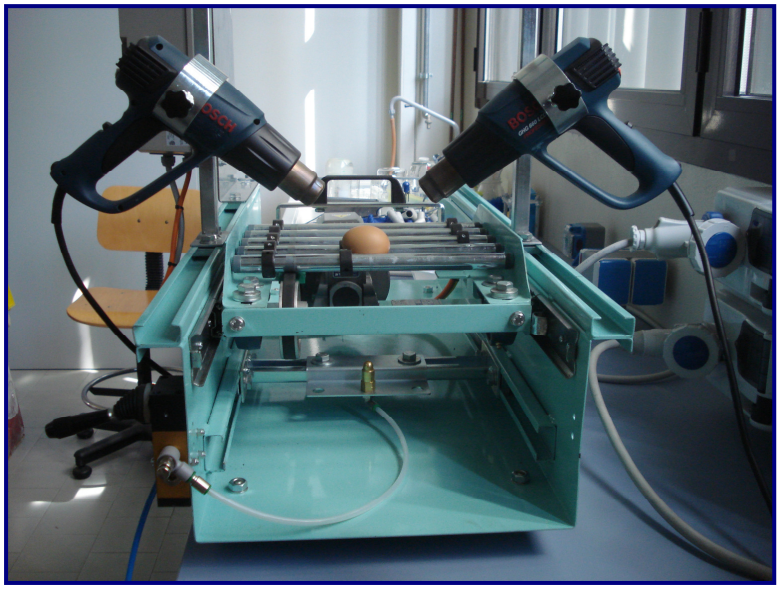

Figure 5.3 – The prototype for hot air treatment of eggs: the two hot air generators, the rolling cylinders for egg support, moved by a transmission belt and, in the lower part, the cold air pipe

The egg was submitted to two shots of hot air for 8 s with an interval of 32 s of air at room temperature. This selected thermal cycle made possible to reach an estimated external eggshell surface temperature higher than 70°C and an inner temperature always lower than 55°C. This hot air treatment was applied on eggs which were experimentally contaminated on the surface with different pathogens (*Salmonella Enteritidis*, *Listeria monocytogenes*,

Escherichia coli) as well as on eggs naturally contaminated by the indigenous microflora and collected from organic poultry farms. The decontamination efficacy was evaluated by detection of cell viability on the eggshell at different days post treatment over a period of shelf life (Pasquali et al., 2010). During the 28 days post treatment the eggs were stored at 20-25 °C temperature.The effect of the treatment on the quality of eggs was evaluated by comparing different quality traits of treated and untreated eggs (Pasquali et al., 2010). These traits were evaluated immediately after the hot air treatment (pH and turbidity of albumen, shell colour and the cuticle assessment) and after a storage of 28 days at 20°C (yolk index, eggshell breaking strength and the shell membranes assessment).

On *S.* Enteritidis and *L. monocytogenes* contaminated eggs, the bacterial load was approx. one log lower in treated eggs in comparison to untreated ones (Figure 5.4). No statistically significant results were obtained comparing *E. coli* on treated and untreated eggs. Since a reduction of 1 log corresponds to a bacterial population reduction of 90%, these results confirm the efficacy of this treatment in reducing the risk for human health due to *S.* Enteritidis and *L. monocytogenes* contamination especially if we consider that the Salmonella load on eggshell is often reported to be approximately no more than 10^2 raising up to 10^3 CFU/ml (or CFU/eggshell) only in rare cases (Humphery et al., 1991) in eggs collected from hens reared in conventional cages.

On naturally contaminated eggs by indigenous microflora, the total mesophilic bacteria load was reduced (approximantely 1 $\log_{10}$ CFU/eggshell) on organic eggs immediately after the treatment. After the third day post treatment and until the end of the storage, the situation was inverted. A statistically significant difference on microbial load was registered with treated eggs being contaminated with the highest load (Figure 5.5). These data might be explained by self adapting metabolic pathway of heat resistant bacteria, which showed an increased growth rate few days after the treatment. This rate, associated with a reduced growth competition, might have induced a higher level of contamination on treated eggs in comparison to untreated ones.

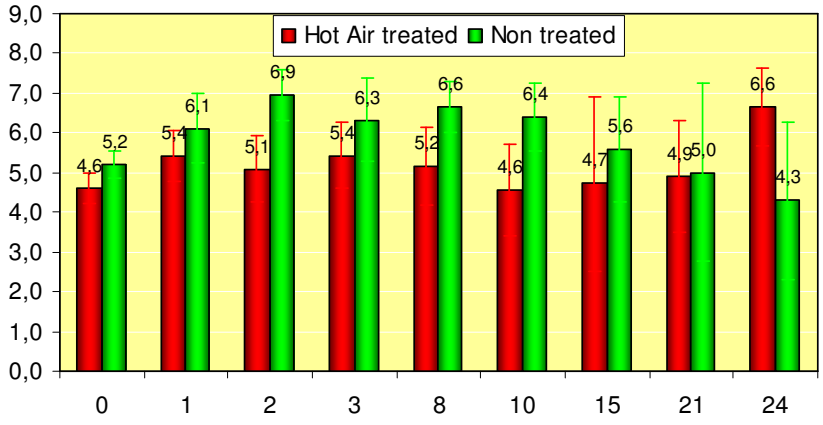

Figure 5.4 - Comparison of S. Enteritidis load ($\log_{10}$ CFU/eggshell) on eggshells of hot air treated and not treated eggs

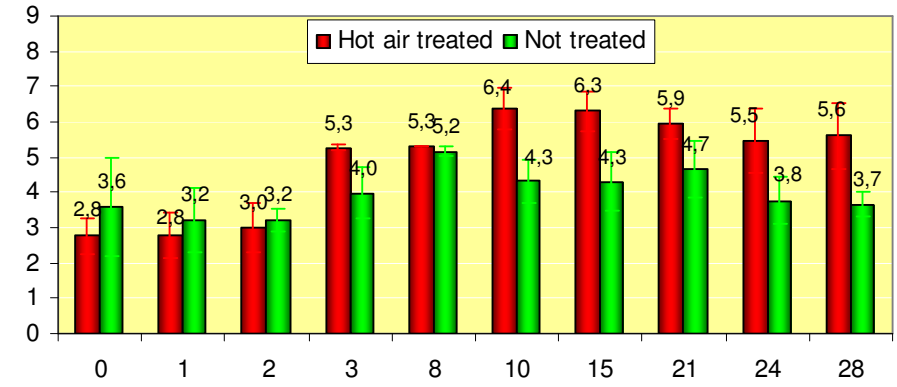

Figure 5.5 - Comparison of indigenous microflora load (CFU/g) on eggshells of hot air treated and not treated eggs collected at an organic poultry farm

All the analysed quality indexes showed no statistically significant differences between treated and untreated eggs, so that it can be assumed that the treatment does not exert negative effects on the main quality traits of egg.
In conclusion, these findings suggest a potential industrial application of the hot air treatment on eggs before packaging for a significant reduction of *S.* Enteritidis, *L. monocytogenes* and total mesophilic bacteria populations which may naturally contaminate the surface of the eggs. However in order to minimize the regrowth of survived bacteria during storage, other approaches need to be identified and coupled to this treatment.

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Decontamination of shell eggs using gas plasma sterilisation

The main objective of the research activities was to assess the applicability and effectiveness of gas plasma technique for the superficial decontamination of shell eggs. Gas plasma is an ionized gas containing electrons and neutral reactive species such as atoms, molecules and radicals, and it is usually produced by exposing a gas (or a mixture of gases) to an electric field that accelerates the charged particles (electrons) producing collisions with the heavy species (Moisan *et al.,* 2001). The UV photons, charged particles and reactive species formed which can act separately or in synergistic combination, are responsible for microbial inactivation (Laroussi and Leipold, 2004). The influence of each factor depends on the operating parameters such as the gas mixture and the voltage used to drive the discharge. This approach was carried out by the University of Bologna.

The specific activities performed included the: (1) identification of the most suitable plasma generation systems for shell egg treatment; (2) set up of three plasma generator prototypes; (3) evaluation of the efficacy of the prototypes for the superficial decontamination of table eggs from the natural microflora and target pathogens (*Salmonella Enteritidis*, *Listeria monocytogenes*, *Escherichia coli, Bacillus cereus* spores and vegetative cells) deliberately inoculated onto the surface of the eggs; (4) evaluation of the ability of the surviving cells (of both the pathogens and the natual microflora) to recover and regrow during a 45 days storage at 25°C in relation to the gas plasma processing conditions, (5) evaluation of plasma side effects on shell egg quality immediately after treatments and during a storage of 28 days at 25°C .

The decontamination power of 3 gas plasma generators was evaluated, by using atmospheric pressure air as working gas, on shell eggs experimentally inoculated with *Salmonella* Enteritidis, *Listeria monocytogenes, Escherichia coli* or *Bacillus cereus*. Figure 5.6 shows one of the analysed prototypes with a detail of the gas plasma discharge.

Figure 5.6 – Resistive Barrier Discharge prototype set up by the Agricultural Economy and Engineering Department, University of Bologna.

The discharge was characterized by the presence of the positive ion N_2^+ indicating the formation of metastables states that are reservoirs of energy promoting plasma chemical reactions and by the presence of OH and NO γ systems radicals.

All the gas plasma generators resulted to be effective against the target microorganisms giving rise to maximum cell reductions ranging between 1.5 and 4 Log

CFU/eggshell following the longest treatments (90 minutes). Significant cell reductions were observed also for the natural microflora immediately after the treatments (Table 5.1).

Microorganism	**Cell reduction (Log CFU/eggshell)**
Salmonella Enteritidis	- 3.8
Listeria monocytogenes	- 4.5
Escherichia coli	- 4.5
Bacillus cereus	
Spores	- 1.5
Vegetative cells	- 1.5
Total mesophiles	- 3.0

Table 5.1 – Cell reductions for different pathogens and total aerobic bacteria onto shelleggs subjected to a 90-minutes gas plasma treatment.

The evolution of the surviving cells during a 50-day storage at 25°C showed the inability of the survivors to regrow and recover the damages caused by the gas plasma treatments. In fact, the mean population detected on both treated and untreated eggs tended to decrease during the storage down to the detection limit due to the inability of the viable or damaged cells to survive during storage in the presence of the hostile conditions that characterize the eggshell surface, i.e. dryness and limited nutrient availability. However, such a tendency was much faster in the eggs subjected to gas plasma than in untreated eggs suggesting that the former are safer for the consumers.

Immediately after the treatments slight significant decrease in the eggshell colour were measured (a*: -7%; b*: -3%). On the contrary, no significant treatment effects were observed in treated eggs in terms of quality of yolk, albumen, shell membranes, eggshell (breaking strength, dynamic stiffness) and cuticle.

The results of the microbiological experiments and the quality traits evaluations obtained suggest that gas plasma can be considered a promising and efficient technique for the superficial decontamination of eggs being able to significantly reduce the population of various pathogens and the natural microflora without affecting the quality of the eggs. Additional advantages of the prototype set up in this work are the use of atmosphere as working gas and the low temperature, which make this equipment of particular interest for industry. On the other hand, the long treatment times necessary to achieve the maximum cell inactivations, which make the use of this prototype not feasible at practical level, could be overcome by more powerful devices of industrial sizes. Gas plasma technique is actually employed for the decontamination of equipments in the healthcare area, re-usable and heat sensitive medical tools, including surface modification for improving wettability and adhesion of polymers (Baier *et al.*, 1992; Dumitrascu *et al.*, 2005).

Another possibility is to couple the gas plasma equipment with other decontamination processes such as intense pulsed-light and photocatalysis. Such associations are particularly promising for plasma-based technologies as they should allow tuning the system: a basal efficacy consuming little energy with the possibility of rapidly turning on high power as required by the degree of contamination (Moreau *et al*., 2008).

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Modified atmosphere packaging (MAP)

Modified atmospheres extend the shelf life of foods by inhibiting chemical, enzymatic and microbial spoilage. The gases used in most MAP applications include CO_2, O_2, N_2 or different combinations of these gases mainly used to preserve fruits and meat during storage. (Gill, 1986; Zagory and Kader, 1988). This task was explored by the University of Bologna.

Within this task the efficacy of MAP in decontaminating shell eggs was investigated together with the possible side effects of the treatment on different quality traits of the egg. Two different MAP technologies were tested for whole egg packaging: under vacuum welding machine and gas flushing welding machine. The first technology was overruled because a too high pressure gradient was generated inside the package increasing the possibility of breaking the eggshell and/or of stressing the internal egg structure. A prototype of gas flushing welding machine was adopted since progressive dilutions of the air in the package head space with modified atmosphere were reached.

The efficacy of three conventional (100% N_2, CO_2 or O_2) and two innovative (100% N_2O or Ar) MA was evaluated for egg preservation during storage at 25 °C in terms of maintenance of qualitative and physical functional properties as well as growth inhibition of *Salmonella* Typhimurium experimentally inoculated on the surface of a representative number of table eggs. Among the gas tested, CO_2 100% was selected as the best gas for egg quality and O_2 100% as the best gas for egg safety. Since the efficacy of each gas on egg preservation is strongly correlated to the temperature of storage, the efficacy of the two gases was then assessed in comparison to air 100% during 45 days of storage at 36°C, 25°C and 4°C on experimentally inoculated eggs with *Salmonella* Enteritidis, *Escherichia coli* or *Listeria monocytogenes* as well as on not inoculated eggs containing only the indigenous microflora and collected from an organic poultry farms.

As far as quality assessment is concerned, in general, eggs packaging permitted a strong reduction of the weight loss of the product and the limitation of pH increase and Haugh unit decrease during storage (Rocculi et al., 2009). As expected, the greatest decline in interior egg quality was observed for the not packed sample, which showed the highest weight loss, pH increase and Haugh unit decrease.

The foam stability decreased during storage for all samples, but eggs packed in 100% CO_2 showed higher foam stability than fresh eggs until the fourth day, reaching values similar to those of the other samples only during the last week of storage.

The hardness of coagulated albumen showed a strict correlation with raw albumen pH; MAP with 100% of CO_2 caused the formation of a soft and puffy coagulum with very low hardness (Figure 5.7) for the all storage period (Figure 5.8), as a consequence of a fast and marked pH reduction and of the expansion of solubilized CO_2 during heating.

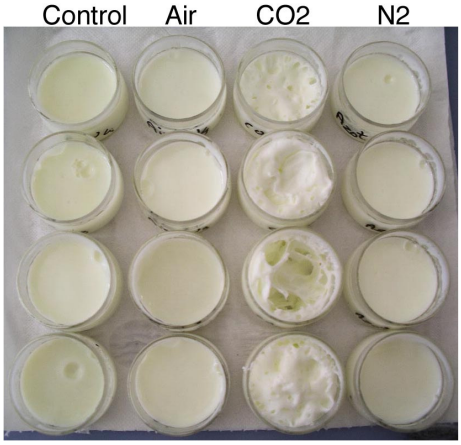

Figure 5.7 - Image of heat coagulated albumen of egg samples after 1 d of storage at 25°C.

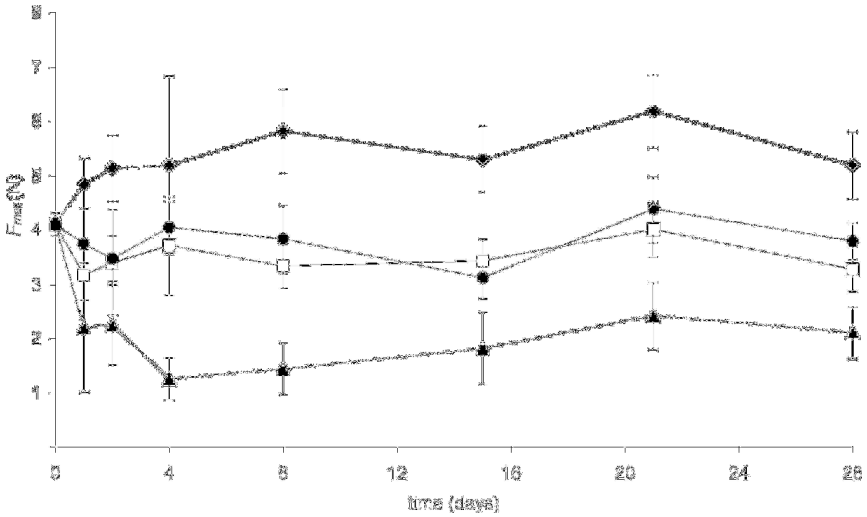

Figure 5.8 - Hardness (F_{max}, (N)) of coagulated albumen cylinders obtained from shell egg samples during 28 days of storage at 25°C. Control (◆): not packed; Air (□): packed in air; N_2 (●): packed in 100 % N_2; CO_2 (▲): packed in 100% CO_2.

From a food safety point of view, overall the initial *S*. Enteritidis and *E. coli* loads on both eggshells and albumens of experimental contaminated eggs increased over the time at 20°C and 36°C whereas they were maintained at 4°C regardless to the gas used for packaging. In particular in the albumen of CO_2 packed eggs, the bacterial loads increased faster than in air and O_2 packed eggs probably because CO_2 acidifies the albumen leading to a decrease of the pH from high values (pH 8-9) to neutral values (pH 7) which represent an optimal pH condition of growth for both *S*. Enteritidis and *E. coli*.

Not surprisingly, for *L. monocytogens* experimental contaminated eggs the results were different in comparison to those obtained for *S. Enteritidis* and *E. coli*. In particular being a psycrotrophic bacteria, as it was expected the initial load of *L. monocytogenes* on both the eggshells and the albumens was maintained over the time at 4°C whereas it decreased at 36°C regardless to the gas used for packaging. In particular at 4 °C the *L. monocytogenes* load was significantly higher in the albumen of O_2 packed eggs in comparison to Air and CO_2

packed eggs These results may be explained by a probable inactivation of the lisozyme in the albumen due to the high oxygen atmosphere. Previous studies on the inactivation effect of oxygen on lysozyme seem to support this hypothesis (Silva et al., 2000).

Regarding the effect of MAP on eggshell and albumen of eggs collected from an organic poultry farm, overall the initial indigenous microflora loads on both eggshell and albumen were maintained over the time at 4°C. At the end of the storage time, CO_2 and O_2 showed the highest decontamination efficacy in comparison to Air at 20 °C and 36 °C respectively with one and three log reductions of the eggshell bacterial load (Figures 5.9). In the albumen the initial microbial load was maintained at all temperatures except for CO_2 packed eggs which showed at 36°C a bacterial load in the albumen two log higher than air packed eggs (Figure 5.10)

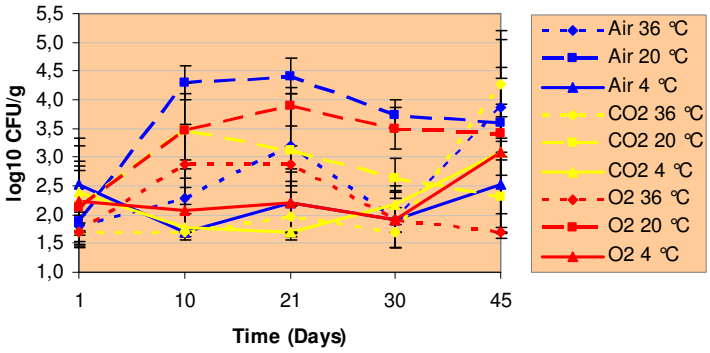

Figure 5.9 - Comparison of the indigenous microflora (total mesophilic bacteria) load (log_{10} CFU/g) on the eggshell of table eggs packed with Air, CO_2 or O_2 and stored at 36, 20 or 4°C at different day of storage

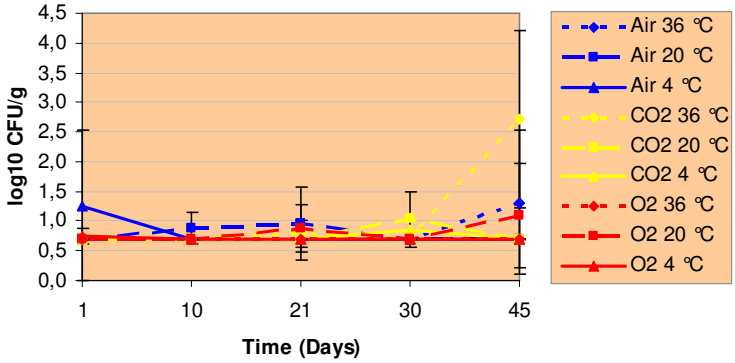

Figure 5.10 - Comparison of the indigenous microflora (total mesophilic bacteria) load (log_{10} CFU/ml) in the albumen of table eggs packed with Air, CO_2 or O_2 and stored at 36, 20 or 4°C at different days of storage

In conclusion from an industrial point of view, the application of this technology (e.g. with controlled atmosphere storage) could permit to improve/modulate the quality characteristics of albumen based food products. In the optic of fresh shell eggs commercialization, CO_2 MAP eggs could represent a tailored innovative food product, with specific characteristics bound to its destination (e.g. fresh shell eggs special for meringue) that could give an added value to the product, both in terms of producer profits and consumer needs. In term of food safety CO_2 at 20°C and O_2 at 36°C were able to show good decontamination efficacies on the indigenous microflora of naturally contaminated eggs.

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Chemical decontamination: coating of eggshell with chitosan

Chitosan is a natural polysaccharide derived by complete or partial (>70%) deacetylation of chitin. Because of its antimicrobial activity and film-forming properties, coating eggshells with chitosan could improve the storability of eggs (as demonstrated by Caner & Cansiz, 2007; Kim *et al.*, 2007), reduce eggshell contamination and prevent or reduce trans-shell penetration by *Salmonella enterica* serovar Enteritidis (SE) and other bacterial strains. This approach was tested for egg by Institute for Agricultural and Fisheries Research (ILVO, Ghent, Belgium)

In our study, first, one chitosan type was selected out of eight types based on its antimicrobial activity towards SE MB1409. Their molecular weights (mw) ranged from 28 to 375 kDa and their deacetylation degrees (dd) were within the range of 75-89.1%. A contact plate method was developed for this purpose. A mean $\log_{10}$ reduction between 0.341 and 0.706 was achieved. We chose the chitosan type with a mw of 310-375 kDa and a dd of 75% because its film was the most flexible of the two chitosan types with the strongest antimicrobial activity. Next, using the same method, the antimicrobial activity of the selected chitosan type towards other strains was tested (see Fig. 5.11). Mean reductions of counts above one $\log_{10}$ unit were obtained for one *Escherichia coli* strain, for *Listeria monocytogenes, Pseudomonas* sp., *Serratia marcescens*, and *Staphylococcus warneri*. Other strains (*Alcaligenes* sp., *Carnobacter* sp., the other *Escherichia coli* strain, and *Salmonella enterica* serovar Typhimurium) had reductions between about 0.5 and 1 $\log_{10}$.

The application of the selected chitosan type as an eggshell coating was then evaluated in comparison to uncoated (control) eggs. Its effect on the shell contamination, trans-shell penetration by SE, and internal quality criteria were included. The eggs were collected from a commercial laying hen flock with conventional cages and housing; the hens were Warren Brown laying hens that were 61-70 weeks old at sampling.

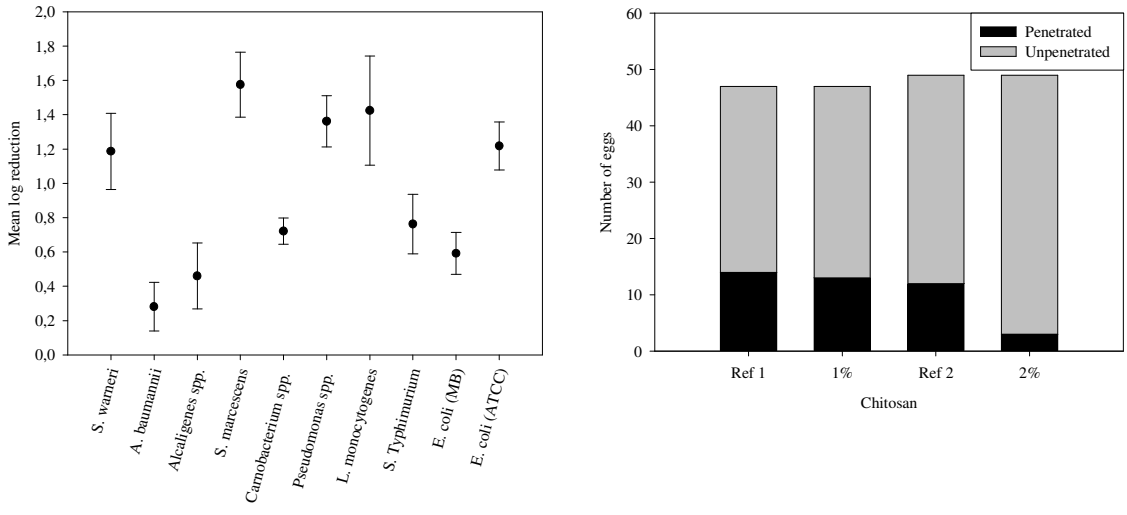
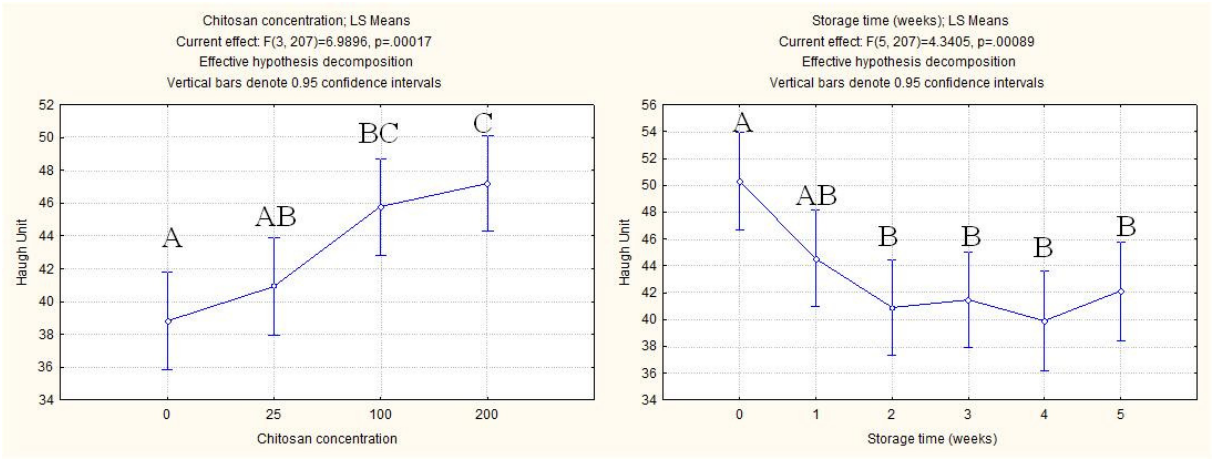


Figure 5.11. The mean log reduction of the egg-related microorganisms and pathogens, as influenced by the selected chitosan type, compared to the reference. The small dot marks the mean. The flags indicate the 95% confidence intervals (left). The number of eggs that were (un)penetrated in the presence of a 1% and 2% chitosan coating, compared to the reference eggs (absence of chitosan; respectively Ref 1 and Ref 2) (right).

To study the shell contamination, three concentrations of the selected chitosan – 0.25%, 1% and 2% – were used for coating. Shortly after coating, the eggshells were inoculated with a mean inoculation dose of 8793 (90% confidence interval: 6835-11 310) cfu per egg. Also eggs were inoculated one week post-coating (0.25% chitosan was used) to evaluate the effectiveness of the coating over time. During three weeks storage at 20°C and 60% relative humidity (RH), the *Salmonella* shell contamination was determined. In general, *Salmonella* counts on the shell declined quickly during storage. After one day of storage, 90.7% of the eggs were still contaminated (with ≥ 1 cell per shell, detection limit), but thereafter this percentage fell to 41.0%, 8.1% and 1.0% after 6, 13 and 20 days of storage, respectively. The decline was similar for the four groups of eggs. Shells coated with 0.25% chitosan one week prior to inoculation showed similar *Salmonella* shell contamination results after 1 day of storage as shells with a fresh coating of 0.25% chitosan. The effect of a chitosan coating on the trans-shell penetration by SE was assessed using the agar approach (see Messens *et al.*, 2005) by which penetration is visible by candling. For this, 1% and 2% chitosan coatings were applied and storage was as above. At day 19 of storage, no significant difference ($p > 0.05$) in percentage of penetrated eggs was observed between eggs coated with 1% chitosan compared to the reference group with 29.8% (14 eggshells penetrated out of 47 tested or 14/47) and 27.7% (13/47) eggshells penetrated, respectively. However, a 2% chitosan coating yielded significant results: a significantly ($p = 0.0058$, one-sided test) lower percentage (6.1%; 3/49) of the eggs was penetrated compared to the control group (24.5%; 12/49).

For internal quality comparison, eggs coated with 0.25%, 1% and 2% chitosan were used and compared to uncoated eggs. Storage was up to five weeks as above. Internal quality criteria included height of the air chamber, the albumen pH, the Haugh Unit (HU), the vitelline membrane strength, the weight loss, the yolk colour and the yolk index. Eggs coated with 1% and 2% chitosan kept their internal quality better than uncoated eggs, as demonstrated by the following examples. The height of the air chamber of eggs coated with a 2% chitosan coating was significantly lower that of both the uncoated eggs and the eggs coated with a 0.25% chitosan coating. During storage, the air chamber becomes larger especially at the beginning of storage. As shown in Figure 5.16, both the eggs coated with 1% and 2% chitosan have significantly higher mean HU values compared to the reference (respectively $p < 0.01$ and $p < 0.001$). During the first two weeks of egg storage, the HU is reduced considerably, but later on, the HU remains stable. Eggs coated with a 1% and 2% chitosan coating have a significantly ($p < 0.05$) higher yolk index compared to the uncoated eggs. Only after two weeks of storage a significant ($p < 0.01$) decrease of the yolk index was observed (Figure 5.12).



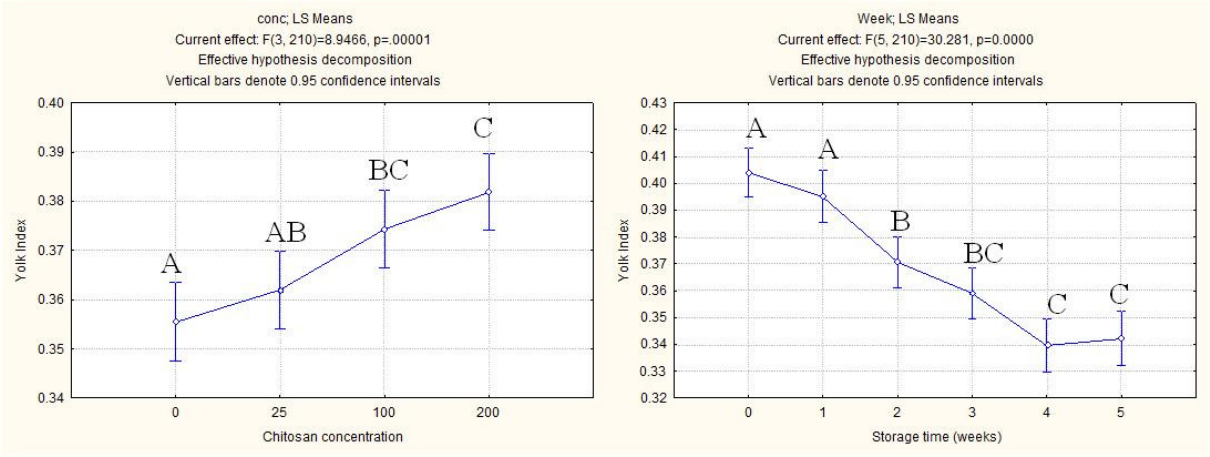


Figure 5.12 The effect of chitosan concentration (left graph; with 0, 25, 100 and 200 indicating the reference, 0.25%, 1% and 2% chitosan concentration, respectively) and storage time (right graph) on the HU values (above) and yolk index (below). Data points within one graph without common letter are significantly different.

In conclusion, the selected chitosan type showed antimicrobial activity towards various egg-related strains and pathogens. On the eggshell, the coating was not proven to be effective to reduce the *Salmonella* shell contamination, but the *Salmonella* counts declined already rapidly during storage in the absence of chitosan. However, a 2% chitosan coating significantly reduced the trans-shell penetration by *Salmonella* and was effective to delay the quality decline of the egg contents. Consumer acceptability of chitosan coatings should be investigated, as the eggs coated with a higher percentage of chitosan generally appeared shinier and smelled somewhat acidic. In future studies, chitosan coating of eggs should be assessed in the field.

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Dissemination of the data issued from RESCAPE

This project was complementary to the SAFEHOUSE project which focused on the influence of the system of egg production on Salmonella dissemination (epidemiological studies) and therefore links were established between both projects especially for dissemination of informations.

In Year 1, the RESCAPE (http://www.rescape-project.eu./) and SAFEHOUSE (http://www.safehouse-project.eu./) websites were launched; an informative booklet summarising the main aims and objectives of the SAFEHOUSE and RESCAPE projects was sent by email to stakeholders (~4500); articles appeared in the European Parliament magazine (Strasbourg) and in the popular poultry press. e.g. World's Poultry magazine. Members of both consortia presented their preliminary findings at key events including the XII European Symposium on Egg and Egg Products in Prague (September 2007).

In year 2, the first SAFEHOUSE and RESCAPE joint newsletter was produced and distributed to stakeholders, ~30 papers were presented at both national and international conferences and meetings including the Worlds Poultry Congress in Brisbane (June '08). A number of peer reviewed papers were also published and several partners contributed chapters to the latest edition of an encyclopedia on eggs (in French : Science et technologie de l'œuf et des ovoproduits, vol.1 and 2 ; Coord : F Nau, C Guérin-Dubiard, F Baron, JL Thapon, Ed Tech et Doc, Lavoisier, Paris, 2010). A joint annual meeting attended by members of both consortia was held in Paris in Sept 08.

In year 3**,** a second joint newsletter was published and sent to ~5000 stakeholders; members of both consortia presented papers at the XIX European Symposium on the Quality of Eggs & Egg Products in Turku, Finland (dedicated session) June 09'. Additional papers were also presented at other key events which included research days and training workshops and a large number (~60) of peer reviewed papers have been published in high impact journals or are in press. Members of both consortia have also submitted chapter plans for the forth coming book entitled **Improving egg and egg product safety and quality. The RESCAPE and SAFEHOUSE coordinators are the editors and the book (42 chapters) will be published by Woodhead Publishing Ltd in 2011.**

A further aim was to source external funds to allow an update to be made to the egg CD-rom on Understanding Egg and Eggshell Quality, which was developed as part of the EU project Egg Defense (2003). Funding to translate the existing CD-rom into other languages was also one of our aims. Unfortunately funding for both translation and updating the existing CD ROM has not been forthcoming. However the successful publication of our new book - **Improving egg and egg product safety and quality-** will ensure that the main outcomes of both the RESCAPE and SAFEHOUSE will be available to a much wider audience through professional advertising and marketing team of Woodhead Publishing Ltd. Both RESCAPE and SAFEHOUSE projects will also receive full recognition for the key role played in the successful publication of this book

This book will be advertised and marketed via our final newsletter (to be published in April 2010), and the website which will remain active until Sept '10. We will also publish a number of articles in the popular press once the final reports of both projects have been submitted. A dedicated session at the forthcoming XIII European poultry congress in Tours in Aug 2010 (www.epc2010.org) chaired by the coordinator of RESCAPE will provide the final platform for show casing the key results of RESCAPE and SAFEHOUSE projects.

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