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Control of *Salmonella* Contamination of Shell Eggs—Preharvest and Postharvest Methods: A Review

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Abstract: *Salmonella* Enteritidis is one of the most prevalent foodborne pathogen, its main reservoir being considered the shell egg. As the concerns related to the increasing human salmonellosis cases grow, the need for an application of preventive methods either at the farm level or during the processing steps is crucial for a better control of the foodborne outbreaks due to the consumption of this specific food product. This review focuses on the application of preventive methods at the farm level, on preharvest step, in order to reduce the risk of shell eggs contamination with *Salmonella*, especially *S.* Enteritidis, through a better control of the laying hens' infection with this pathogen. As postharvest methods, a 1st approach is the egg storage conditions and the prevention of *Salmonella* spp. growth and multiplication. In addition, shell eggs may be subjected to eggshell decontamination, to reduce the risk of foodborne outbreaks. Several of these latter mentioned methods are already authorized to be put in place in different countries, as it is the case in the United States of America and Canada. Their efficacy has been proven and their use is regarded by some as mandatory for ensuring shell eggs safety for the consumers.

Introduction

Salmonella genus is amember of the *Enterobacteriaceae* family, comprising Gram-negative rod-shaped nonspore-forming bacteria. Their main reservoir is the intestinal tract of humans and animals (Bhunia 2007).

Among the different serotypes of *Salmonella enterica*, *S.* Enteritidis, and *S.* Typhimurium account for the most nontyphoidal *Salmonella* infections in both developed and developing countries (CDC 2010, 2012; EFSA 2010; Majowicz and others 2010; Scallan and others 2011; Wales and Davies 2011; EFSA 2012).These serotypes are regarded as unrestricted, being able to cause infections in animals as well as in humans (Martelli and Davies 2012). Eggs and egg-based products were frequently associated with salmonellosis outbreaks caused by *S.* Enteritidis in the United States of America (U.S.A.), as well as in the European Union (E.U.) (Braden 2006; EFSA 2012). This is a potential consequence of the high frequency at which *S.* Enteritidis colonizes the ovaries of laying hens (Gantois and others 2008). Usually this happens without any lesions and furthermore, when egg storage conditions allow it, this foodborne pathogen may be isolated from the shell egg, as it survives in the forming egg (Gast and others 2007; Gantois and others 2009; Raspoet and others 2011; Howard and others 2012). Transmission of this serotype may happen either vertically (Gast and Beard 1990; Galàn 2001;Groisman 2001; Gast and others 2002; Gast and others 2004; Gyles and others 2004; Gast and others 2007; Ibarra and Steele-Mortimer 2009; Li and others 2009; Mastroeni and others 2009; Dawoud and others 2011; De Vylder and others 2011; Desin and others 2011; Linke and Goldman 2011; Shah and others 2011; Howard and others 2012; Kumar 2012) or horizontally (Holt 1995; Holt and others 1998; Jones and others 2002; Davies and Breslin 2003b; De Reu and others 2006; Musgrove and others 2012).

In comparison to *S.* Enteritidis, *S.* Typhimurium is less frequently encountered to be a cause of human salmonellosis due to consumption of shell eggs. However, its ability to colonize the reproductive tract of the laying hens and contaminate the forming eggs has also been determined (Okamura and others 2005; Wales and others 2007; Gantois and others 2008; Okamura and others 2010; Wales and Davies 2011 Martelli and Davies 2012).

Other serotypes of *S. enterica*, such as *S.* Infantis, *S.* Virchow, *S.* Heidelberg are very rarely found to be contaminating shell eggs (CDC 2010).

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The preventive methods for reducing the risk of *Salmonella* contamination of shell eggs and human salmonellosis outbreaks due to their consumption can be either applied as preharvest or as postharvest procedures. Furthermore, they can be either serotype-specific or serotype-independent, the latter being considered a more complex approach (Gast 2007). The environment of the laying hen house can act as reservoir for *Salmonella* (Henzler and Optiz 1992; Davies and Wray 1995, 1996; Eriksson de Rezende and others 2001; Davies and Breslin 2003b; Davis and Morishita 2005; Holt and others 2007; Umali and others 2012), along with the feed, that can be already contaminated as it arrives in the farm (Davies and Hinton 2000; Shirota and others 2000; Maciorowski and others 2006; Gast 2007; Davies and Wales 2010). Due to these various sources of infection for the laying hens, preventive methods are already applied or available at the farm level: flock testing, sanitation and biosecurity (Gast and Beard 1990; Davison and others 1996; Hogue and others 1997; Davies and Breslin 2001; Gast 2007; Arnold and others 2010; Gast and Guard 2011; Holt and others 2011); vaccination (Nakamura and others 1994; Liu and others 2001; Goldsby and others 2003; Khan and others 2003; De Buck and others 2004a; Van Immerseel and others 2005b, 2005c; Gantois and others 2006; Toyota-Hanatani and others 2009; Omwandho and Kubota 2010); passive immunization (Gürtler and others 2004; Chalghoumi and others 2008, 2009a, 2009b); the use of natural antimicrobial products such as bacteriophages (Joerger 2003; Toro and others 2005; Borie and others 2009; Monk and others 2010; Waseh and others 2010), protein and fiber sources (Sugita-Konishi and others 2002; Kassaify and Mine 2004a, 2004b, 2005), competitive exclusion flora, probiotics, prebiotics, and organic acids (Schneitz and Mead 2000; Seo and others 2000; Tellez and others 2001; Van Immerseel and other 2002; Schneitz 2005; Van Immerseel and others 2005a; Doyle and Erickson 2006; Lima and others 2007; Sterzo and others 2007; Van Coillie and others 2007; Van Immerseel and others 2007; Donalson and others 2008b; Vandeplas and others 2010; Tellez and others 2012), essential oils (Chao and others 2000; Lee and others 2004; Johny and others 2008; O'Bryan and others 2008; Brenes and Roura 2010; Ouwehand and others 2010), and bacteriocins (Cleveland and others 2001; Gordon and other 2007; Heng and others 2007; Dias Paiva and others 2011). For postharvest control of *Salmonella* in shell eggs, the 1st approach is to maintain an adequate temperature during storage (Gast and Holt 2000, 2001; Gast and others 2006; Lublin and Sela 2008; FDA 2009a, 2009b; Gantois and others 2009). However, different surface decontamination methods are already applied in the U.S.A. and new ones make the subject of continuous research: egg washing (Hutchison and others 2003; Jones and others 2005; Caudill and others 2010); electrolyzed water (Huang and others 2008; Howard and others 2012; Mukhopadhyay and Ramaswamy 2012);ozone (Davies and Breslin 2003a; Rodriguez-Romo and others 2007; Perry and others 2008); ultrasounds (Cabeza and others 2011); microwaves (Lakins and others 2008); irradiation (Serrano and others 1997; Wong and Kitts 2003; Cabo Verde and others 2004); gas plasma (Kayes and others 2007; Ragni and others 2010); ultraviolet light (Rodriguez-Romo and Yousef 2005); and pulsed light (Hierro and others 2009). Among all these, the ones authorized in the U.S.A. are the shell washing and irradiation (USDA 2005; FDA 2009b).

The aim of this paper is to review most of the postharvest and preharvest methods for reducing *Salmonella* contamination of shell eggs and furthermore the risk of human salmonellosis outbreaks caused by this food product.

Preharvest Methods for Reducing the Risk of *Salmonella* Contamination of Shell Eggs

Salmonella carrier state in poultry and the genetic control of resistance to salmonellosis

Among the different preventive methods used against *Salmonella* in laying hens, genetic selection may be a promising one in reducing the occurrence of salmonellosis in layers. It has been shown, indeed, that genetic lines of laying hens exhibit different resistance levels against *Salmonella* spp. (Gantois and others 2009).

A genetic correlation between *Salmonella* spp. contamination level in different tissues has been demonstrated. In an investigation concerning the heritability of resistance trait in laying hens, Girard-Santosuosso and others (2002) demonstrated that the genetic correlation (r) between the concentration ($\log_{10}$ CFU/g) of *S.* Enteritidis in the liver and the genital organs was high (0.56). A similar result was found for the concentration of *S.* Enteritidis in the spleen and in the genital organs, with a correlation of 0.79. The authors suggested that the genes controlling the contamination of these organs are the same. Beaumont and others (2009a) estimated that in adult laying hens the genetic correlation between global contamination and ovarian contamination was 0.32, while between global contamination and the other organs it was high: 0.75 for the liver and, 0.85 for the spleen and ceca, with a probability of 100% of being positive.

The assessment of genetically regulated resistance is of high importance for genetic control of resistance to *Salmonella* spp. infection. The studies investigating this subject were all aimed to reduce the disease occurrence and economic losses in laying hens, as well as controlling *Salmonella* spp. colonization of internal organs and the contamination of their products, especially focusing on *S.* Enteritidis and *S.* Typhimurium (Wigley 2004). In a study performed by Sadeyen and others (2006), 2 inbred lines of laying hens known for different resistance traits were orally inoculated with *S.* Enteritidis. Bacterial colonization and the host gene expression were measured in the ceca and the gut-associated lymphoid tissue. The expression of chemokine, of the anti-infectious cytokine, of the bacterial receptor, of the antimicrobial mediator, and, particularly, of the defensin genes were all increased in the line carrying a lower level of bacteria in the ceca. These innate immunity molecules were either constitutively or inductively highly expressed in resistant adult birds, thus being considered candidate genes that could play a role in a host's protection against *Salmonella* colonization. In a previous study, Sadeyen and others (2004) revealed that susceptible lines expressed a lower baseline level of IFN-γ than resistant lines. They concluded that the persistence of *Salmonella* spp. in the digestive tract is caused by an immunodeficiency state.

Considering the systemic phase of infection, Fife and others (2009) showed that resistance is partly determined by genetic strains and that, in resistant lines, the microbial load can reach values of up to 1000 times lower, in comparison with susceptible lines. The identification of genes contributing to resistance against this disease may therefore enhance the genetic selection of the resistant lines. Furthermore, Prévost and others (2008) revealed that in experimental conditions the crossbreeding between different selected lines, for lower or higher propensity to carry *Salmonella* spp., resulted in a reduction by half of the maximal percentage of contaminated animals. Nevertheless, they were unable to accelerate the extinction of disease.

As stated above, the level of resistance differs from one line to another. However, inside a particular line, age has been shown to influence the genetic control of resistance. This may be linked directly to the mechanisms of resistance, chickens being only

protected by the innate immune response, while adult hens may also benefit from the adaptive immune system (Beaumont and others 2009b). The chicken antibody repertoire is generated during the late embryonic stage and for a short period after hatching. As the chick ages, its B cells undergo additional rounds of somatic gene conversion and its antibody repertoire achieves a mature state around the age of 5 to 7 wk. This corresponds to the age the bursa becomes fully mature (Davidson and others 2008).

Resistance to systemic salmonellosis in poultry is encoded by a number of factors, several of them being of genetic nature. The gene *Slc11a1* was 1st identified in mice (Roy and Malo 2002). Its physiological and functional properties support its role in controlling the intracellular replication of foreign microorganisms in phagosomes. *Slc11a1* alleles were shown to be involved in early as well as in late resistance. The effect of the *Slc11a1* locus has been moreover associated with the carrier-state resistance variations in divergent chicken lines (Wigley 2004; Calenge and others 2010).

Another resistance-related factor called Toll-like receptor 4 belongs to a family of innate immune system receptors involved in the recognition of lipopolysaccharides (LPS) from Gram-negative bacteria (Calenge and others 2010; Chaussé and others 2011).

Recent mapping has revealed a new locus on chicken chromosome 5, accounting for a major part of the differences in susceptibility between the lines (Fife and others 2009). This novel gene has been named *SAL1* as it seems to play a role in increasing macrophage activity against *Salmonella* spp. The differences in the pathology of infection between the resistant and the susceptible lines indicate that the key to the resistance lies within the mononuclear/phagocytic cell function. *SAL1* locus was assessed by high-density single nucleotide polymorphism (SNP) panels in combination with backcrossing of the resistant and susceptible lines and, after refining, it has been shown that this region spans 14 genes, including 2 striking functional candidates: CD-27 binding protein (Siva-1) and the RAC-alpha serine/threonine protein kinase homologue AKT1. Siva-1 is an apoptosis-inducing factor, which possesses the ability to activate the process of induced cell death and to downregulate the immune response. AKT-1 activates NF-κB via regulation of IκB kinase, resulting in transcription of prosurvival genes, which are directly involved in *Salmonella*-induced apoptosis (Fife and others 2009).

Besides selection, genetic engineering has also been investigated as an alternative strategy to traditional animal crossbreeding. The goal is the enhancement of an animal's ability to develop an appropriate immune response against the pathogen. However, even when the desirable allele for resistance is present in a population, it may be difficult to introduce it into a given genotype due to the simultaneous introduction of many unrelated and unknown traits. Once genetically modified animals exhibit the trait of resistance to *Salmonella* spp. they could be introduced into the breeding stock. Nevertheless, their introduction in the food chain is still controversial (Whitelaw and Sang 2005).

Until now, all the identified genes and loci concerning laying hens' resistance to *Salmonella* spp. represent potential subjects for further investigation on the possibility of selection for this specific trait. The improvement of laying hens' genetic resistance to *Salmonella* spp. carrier-state could represent a complementary way to reduce *Salmonella* spp. propagation, thus enhancing the possibility for other preventive methods to succeed in reducing the rate of contamination with high-risk *Salmonella* serotypes such as *S.* Enteritidis and *S.* Typhimurium.

Before taking into account the possibility to use the result of genetic studies considering the improvement of resistance against *Salmonella* in laying hens, it is certainly necessary to confirm the roles of the different investigated genes and resistance factors.

Beaumont and others (2010) suggest that even when choosing an approach by candidate genes, the association observed between the quantitative trait loci region and the character encoded does not allow excluding the possibility of another gene's involvement. The most detailed study was the one investigating *SAL1*, this permitting to reduce the quantitative trait loci presence zone to a region, which contains only 14 genes (Fife and others 2009). If continued, the efforts will lead to an obvious development in the field of SNP methods, to a larger scale, permitting the progress in different rapid applications.

Flock management

The high incidence of human salmonellosis caused specifically by *S.* Enteritidis through the consumption of contaminated shell eggs or egg products derived from such contaminated shell eggs recently determined the development and implementation of multiple *Salmonella* programs. These comprise a series of testing and monitoring methods, as well as several procedures (including cleaning and disinfection, control of pests) considered very efficient in reducing the risk of *Salmonella* presence in the environment of the hen houses.

Gast (2007) considers that a more serotype-independent approach for reducing the risk of shell eggs contamination with *Salmonella* has the advantage of detecting and responding to emerging problems before their impact becomes more severe. The same author concludes that, regarding the preharvest *Salmonella* control programs in poultry flocks, no single type of response (a serotype-specific one or a serotype-independent one) could provide a unilateral solution to the complex public health and economic problems related to this foodborne pathogen.

A series of environmental-related factors may influence the likelihood and outcome of *Salmonella* infections in poultry. These factors are: litter, dust, mice, flies and the different surfaces from the hen houses or the farm, with which the laying hens may come in contact with. Davies and Breslin (2003b) showed that during a 26-mo postdepopulation period, with periodical sampling from the environment of a free-range breeding farm, *S.* Enteritidis Phage Type 4 (PT4) was persistently present in the soil, in the litter, nest boxes, feed troughs and mice droppings.

The levels of *Salmonella* in the litter have been reported to increase with increasing the water-activity levels and the moisture content, mostly due to accidental water leakage (Eriksson de Rezende and others 2001). For this, preventive methods are applied, such as maintaining a litter drying environment through a modest and uniformly distributed ventilation rate (100 to 150 ft/min) over the litter surface. Turnbull and Snoeyenbos (1973) observed that a high pH value of the litter, caused by the ammonia dissolved in the available moisture of the litter, was unfavorable for *Salmonella* growth. Also, Bennett and others (2003) observed that addition of hydrated lime to the litter can markedly reduce *Salmonella* Enteritidis recovery in a relatively short time (< 24 h), due to the increase in pH of up to 12.57 at an addition of 20% of lime.

Dust has been associated with a long persistence of *Salmonella* in the poultry houses. Davies and Wray (1996) observed that *S.* Enteritidis was frequently found surviving in small pockets of fan dust, which had been left after cleaning and disinfection of the poultry house. This result came from a study on the survival of *S.* Enteritidis in poultry units carried out over a period of 2 y. Also, it appears that *S.* Enteritidis persists preferentially when

associated with dust particles swept from the floor and the feed troughs, with at least a 26-mo survival in artificially contaminated poultry feed. Davis and Morishita (2005) found that *Salmonella* spp. could be isolated as an airborne pathogen, inside the laying hens' house, and as well in the outside area of the hen house, up to a 40 ft distance (approximately 13 m). Dust could possibly act as a vector for *S.* Enteritidis spread from infected hens to healthy ones, through a potential airborne transmission. Gast and others (1998) studied the mechanism by which *S.* Enteritidis might spread between groups of chicks housed in controlled-environment disease transmission cabinets. The airflow was directed from one group ("upstream") to another ("downstream"). Groups consisting of 25 1-d-old chicks were placed in the upstream ends and orally inoculated with *S.* Enteritidis. One day later, another group of 25 1-d-old chicks was placed in the downstream end. At 3 and 7 d postinoculation, *S.* Enteritidis was found on the feathers of 77% of the downstream chicks, among them 33% already infected with *S.* Enteritidis. The authors suggested that the infection was apparently transmitted through oral ingestion, probably from environmental surfaces contaminated by airborne movement of the pathogen. This led to the conclusion that a reduction of *S.* Enteritidis airborne-movement would limit the spread of infection within flocks, further on reducing the incidence of potentially contaminated eggs.

Due to the wide host range of S*almonella* spp., different biologic vectors may appear and pose a risk for an infection in poultry, through the dissemination of the pathogen. Mice are considered the main reservoir among the biologic vectors, Henzler and Opitz (1992) revealing that the bacterial count from the feces of one mouse can yield 2.3×10^5 *S.* Enteritidis bacteria/fecal pellet. Moreover, this serotype can persist up to 10 mo in an infected mice population. Persistent *S.* Enteritidis infection in the poultry units are usually a result of a high proportion of mice found to carry this pathogen. Davies and Wray (1995) showed that *S.* Enteritidis determines a systemic infection in mice. At the farm level, 3-wk-old chicken were infected with *S.* Enteritidis through direct contact with droppings from mice experimentally infected 5 mo previously. In addition, wild mice infected artificially or naturally, excreted *S.* Enteritidis intermittently, with up to 10^4 CFU/individual dropping.

Rats, as mice, are considered a reservoir of *Salmonella*, with a high risk of poultry infections. Umali and others (2012) studied the transmission and shedding patterns of *Salmonella* in naturally infected wild rats, through daily observations and sampling. *S.* Enteritidis was more frequently isolated from the spleen and liver at the end of the study, in comparison to the number of positive cultures from the feces. Moreover, the authors isolated another serotype, *S.* Infantis, which determined more likely an enteric type of infection. This was due to a much higher frequency of its isolation from the feces, while absent in the organs.

Insects could also be considered a vector of *Salmonella*, one of the most frequently encountered muscoid flies being *Musca domestica*, also called the housefly. Mian and others (2002) found that among the muscoid flies, at commercial farms subjected to tests for *Salmonella* Enteritidis presence, 5 species were encountered, and among these, the housefly was the only one tested positive for *S.* Enteritidis. Further on, Holt and others (2007) demonstrated that flies exposed to an environment containing *S.* Enteritidis can become colonized with the microorganism and might serve as a source for transmission of *S.* Enteritidis within a flock situation. Flies collected at caged-layer facilities were involved in 2 outbreaks of *S.* Enteritidis infections due to contaminated shell eggs. Among the existing flies, houseflies were *S.* Heidelberg and *S.* Enteritidis carriers (2 out of 15 pooled samples for the latter serotype) and dump flies (*Hydrotaea aenescens*) were carriers of *S.* Infantis (Olsen and Hammack 2000).

Related to flock management, Holt and others (2011) mention that one of the factors that can affect the prevalence of *Salmonella* on premises is the flock size. A potential connection between the high stocking density of laying hens in conventional cages and the large volume of feces and dust may lead to an increase in the incidence of *Salmonella* infections in this particular type of housing system (Davies and Breslin 2004). In addition, high stocking densities may indirectly interact with *Salmonella* infections because of the caused stress (Van Hoorebeke and others 2011).

Feed management practices and foodborne *Salmonella* spp. contamination of poultry feeds

Feed withdrawal for molting purposes. Molting induced by feed withdrawal, a common practice destined to increase egg productivity and decrease hen mortality (Alodan and Mashaly 1999), has been shown to enhance the risk of vertical transmission of *Salmonella* spp. (Holt 1999; Golden and others 2008). Berry (2003) states that during the induced molting, due to stress, transient reductions in the number of specific lymphocyte classes appear, which may cause an increased susceptibility to infection. The same author states that reduced mortality during and after induced molting suggests that this practice does impair the immune function with respect to avian pathogens, but only to a limited degree. In addition, during the molting periods, *S.* Enteritidis can be transmitted to uninfected layers from infected ones (Holt 1995; Holt and others 1998).

Durant and others (1999) showed during a challenge with S. Enteritidis (10^5 organisms) that through feed deprivation, applied as a method for molting, the numbers of lactobacilli and the concentrations of lactate, acetate, propionate and butyrate as well as the total volatile fatty acids (VFA) in the crops decreased, while crop pH increased. As a result, crop and cecal colonization of S. Enteritidis increased significantly in molted hens compared to the controls. This suggests that the changes in the crop environment, caused by feed deprivation are important for the regulation of S. Enteritidis survival.

For this reason, research has aimed to develop alternative methods for molting, new procedures that would avoid feed removal, but retain at the same time the economic benefits (Maciorowski and others 2006). For molting purposes, Woodward and others (2005) demonstrated that alfalfa could be used as an alternative, resulting in a reduction of *S.* Enteritidis colonization in experimentally challenged laying hens. Furthermore, in order to decrease the population of *Salmonella* spp. in the ceca of laying hens during molting, Willis and others (2008) assessed a combination of alfalfa and an extract of *Lentinus edodes,* also known as the Shiitake mushroom (Leatham 1982). The results showed a high decrease, up to 2.72 log CFU/g from the initial *Salmonella* spp. counts, suggesting that this combination might be successfully used as an alternative to feed removal during molting periods.

Feeding laying hens with wheat middlings caused a cessation of egg production within 3 to 7 d. The comparison of *S.* Enteritidis levels between unmolted group, molted by feed withdrawal group and wheat middlings feeding group resulted in a difference of 3 to 5 log more *S.* Enteritidis in the feed withdrawal group (Seo and others 2001).

Whole cottonseed meal (50% of the diet) can also be used when inducing molting, hens voluntarily reducing their feed intake. This type of molting is believed to be equivalent in effectiveness to the one produced by complete feed withdrawal, and with the same consequences on the egg safety, by increasing the risk of *S.* Enteritidis contamination (Davis and others 2002). Induction of molt by using grape pomace freely for 10, 14, or 18 d resulted in a postmolting performance comparable to that of hens exposed to 10 d of feed removal.

Keshavarz and Quimby (2002) observed that layers on continuous feed removal and grape pomace with added thyroxin went out of production 3 to 4 d after the initiation of molt. Furthermore, egg production for 70 to 98 wk of age or 66 to 98 wk of age, as well as egg mass and feed conversion were similar for feed removal and grape pomace plus thyroxin treatments.

Foodborne *Salmonella* spp. contamination of poultry feeds. Poultry feed can become contaminated with foodborne *Salmonella* either during harvesting, processing at the feed mill or storage (Maciorowski and others 2006). Poultry feeds can also become contaminated with salmonellae from animal proteins and other ingredients, or even from the dust present in the feed mills (Gast 2007).

Salmonella contamination of complete animal feed seems to be common, as studies from the U.S.A., as well as from several European countries, report *Salmonella* contamination rates in complete animal feed (finished feed) that range from 1.1% to 41.7% (Li and others 2012).

In Japan, Shirota and others (2000) isolated 148 strains of *Salmonella* spp. from 143 out of 4418 feed samples subjected to analysis. The isolated strains consisted of 32 serotypes, including 20 strains of *S.* Enteritidis, mainly originating in samples collected from feed mills. Davies and Wales (2010) investigated 4 commercial feed mills and 4 on-farm poultry feed mixers for the presence of *Salmonella* spp. They revealed that the serotypes (among them none pertaining to *S.* Enteritidis) present in raw feed ingredients on farms were associated with wildlife and/or livestock, whereas those in commercial mill ingredients were associated with home-produced cereals and imported vegetable protein sources. The ingredient contamination, particularly of cereals, may be attributed to wildlife, such as badgers and rodents that defecate in crops or in storage facilities (Davies and Hinton 2000).

Different protein sources and cereals have been identified as contaminated with *Salmonella* spp.: peanut meal, sunflower meal, soybean meal, bran meal, barley, corn, sorghum, and wheat (Maciorowski and others 2006). Sunflower yielded the highest number of positive samples for *Salmonella* spp. presence (MacKenzie and Bains 1976).

Animal protein and byproducts destined for obtaining protein meals for animal feed have always been considered a major source of *Salmonella* spp., one cause being the incomplete decontamination of these ingredients during processing (Davies and others 2004).

Among these animal protein sources, meat meals (Mackenzie and Bains 1975; Hacking and others 1978; Nabbut 1978) and feather meals (Hacking and others 1978) have already been triggered as *Salmonella* sources. In addition, bone meal and fishmeal are apparently also sources of contamination with *Salmonella* (Nabbut 1978). In a study conducted on the Dutch feed industry, mash feeds used for layers were more frequently contaminated than pelleted ones, suggesting the role of the increased temperature during pelleting for a suitable reduction of the foodborne pathogens. Considering their nature, 31% out of 130 fishmeal samples were contaminated in comparison to 4% out of 83 meat and bone meal samples. Therefore, fishmeals may have the tendency to be more frequently contaminated with *Salmonella* spp. than other types of animal protein sources (Veldman and others 1995).

During the feed processing, due to different processes to which the ingredients are subjected to (grinding, mixing, and pelleting), *Salmonella* spp. contamination can occur, as well as recontamination, once the processing is over.

According to Whyte and others (2003), *Salmonella* may be present in preheat as well as in postheat treatment areas of the poultry feed mills. They recovered the pathogen from samples of feed and dust, with overall percentages of 18.8% and 22.6%, respectively. A percentage of 11.8% of feed samples collected from a preheat area were associated with *Salmonella* presence, while from the same area, 33.3% of the dust samples were also *Salmonella* positive. From the postheat areas, 24.2% of the dust samples were *Salmonella* positive. In addition, the feed delivery area was considered a *Salmonella* recontamination space, therefore, samples were collected from this site too. It appeared that 57.1% of the samples taken from it were *Salmonella* positive.

At the level of primary production of feed, the ingredients obtained from different vegetal sources may become contaminated with *Salmonella* following direct contact with fertilizers. This could be reduced through different measures, as the EFSA report on the microbiological risk assessment of feedingstuffs mentions: storage of the fertilizer more than 2 mo, without any new influx; composting; ploughing in after spreading the fertilizer; increasing the time allowed between spreading of the fertilizer and the animal grazing or crop harvesting, heat treatment of fertilizers before use and treating fertilizers by lime addition. The conditions of transport and storage of the ingredients are considered of high importance, the risk increasing with the poor hygiene and no respect of good practices (EFSA 2008).

Salmonella control principles involve preventing contamination from entering the facility, reducing multiplication within the plant and killing the pathogen. Among the preventive measures to be applied for *Salmonella* feed contamination, the most important are obtaining *Salmonella*-free feed ingredients (Jones 2006), controlling the dust (Whyte and others 2003; Jones and Richardson 2004), restricting the flow of the personnel (EFSA 2008), reduction of fat accumulation, controlling rodents and wild birds and maintaining the sanitation of the transport vehicles (Fedorka-Cray and others 1997).

Maciorowski and others (2007) suggested different methods for controlling *Salmonella* contamination in feed. For feed degradation, shortening storage time to prevent browning and caking of the feed and the supplementation with soybean oil to prevent fat losses would be of 1st importance, before implementing other prevention methods. In addition, rapid drying is widely used to preserve raw feed ingredients (ICMSF 2005). Considering the addition of different antimicrobial agents, disinfectants such as acids (mineral acids, short-chain fatty acids), isopropyl alcohol, aldehydes, and trisodium phosphate may reduce the risk of contamination with *Salmonella,* through inactivation of this pathogen during the storage of feed (Maciorowski and others 2007). However, the same authors conclude that the efficacy of these additives may be reduced, due to the high concentration of organic matter in the feed. Moreover, several of them may act as corrosives and/or are toxic when introduced in high concentrations. Therefore, their use should be limited in processed feed.

Inactivation of *Salmonella* in feeds may involve pelleting (which consists of thermal processing) and/or chemical addition. The pelleting process consists of 3 major steps: mixing steam with mash feed (also called conditioning), pressing conditioned feed through metal dies (pelleting), and removal of heat and moisture via large volumes of air (cooling). During conditioning, it is considered that an amount of 10^3 CFU/100 g is destroyed, with destruction beginning at approximately 71 °C (Jones 2011).

Data presented by Jones and Richardson (2004) indicate that 16 out of 178 samples of mash diets (8.79%) were positive for *Salmonella*, while 19 out of 451 samples of pelleted feed (4.21%) were contaminated. This suggests that the pelleting process reduced the number of *Salmonella* cells isolated from feed by 50%.

Fancher and others (1996) reported that the use of an expander for conditioning step has, among others, the advantage of ensuring lower feed moisture, therefore an increased feed hygiene. The expander is a device that resembles to a single-screw extruder, which, in change, discharges the feed over an annular gap, instead of forcing it through a fixed die. This expander may improve the hygienic quality of feed, through a reduction of bacterial loads by 10^5 to 10^6 CFU/g. This is accomplished through values of temperature of 115 to 125 °C and pressure of up to 1200 psi, maintained for 10 to 20 s (Fancher and others 1996).

Steaming during pelleting process has been shown to eliminate bacteria (Stott and others 1975; Furuta and others 1980a,b). Stott and others (1975) showed that pelleting process reduced up to 1000-fold the numbers of *Enterobacteriaceae* in poultry feed, by sampling before and after this processing stage. Also, they isolated *Salmonella* only from samples of meat and bone meal. Furuta and others (1980a) observed that mash diets treated at 70 to 80 °C for 5 s in order to obtain pellets and crumbles, suffered a reduction of the number of bacterial colonies from 1.9×10^3 to $3.0 \times 10^{\circ}$ CFU/g.

The success of reducing *Salmonella* colony-forming units in feed may be influenced by different factors, related to the physical quality of the pellets, as they are discussed by Thomas and van der Poel (1996) and Thomas and others (1997, 1998). Apparently, different properties of the ingredients influence directly the binding process, with further influence on the pellet quality, evaluated through hardness and durability. In addition, the equipment plays a very important role in the final quality, with a direct influence on the final hygienic characteristics of the feed.

After conditioning and heat treatment, the cooling step poses a great risk of recontamination with *Salmonella*, in 2 ways. During cooling, condensation may occur if the temperature difference is more than 5 °C between the pelleted feed and the environment. Therefore, the warm pellets will determine condensation and free water in the so-called "clean-side" of the feed manufacturing facility. Condensation droplets, in favorable conditions, may lead to *Salmonella* growth, either on the top of the conveyor or in the silo. To reduce the microbiological risk that this problem may pose, the insulation of the top of the cooler can reduce the risk of condensation (EFSA 2008). Moreover, because pellet coolers pull large volumes of air, dust obtained from the cooler would appear to have a greater likelihood of contamination than dust collected in other areas. In addition, mechanical vibrations and air currents around the pellet mill may result in dust particles being dislodged and falling into the pelleted feed (Jones and Richardson 2004). EFSA (2008) mentions that adequate dust collector systems in the feed manufacturing facilities are important to control dust and to keep the environment in a clean condition.

Flock testing, sanitation, and biosecurity

Testing is a very important part of the *Salmonella* control programs. Testing is however controversial as its efficacy may be sometimes low, due to a continuous reintroduction of many serotypes of *Salmonella* (including *S.* Enteritidis) in the poultry houses and flocks, from environmental sources. Trace-back testing has generally not been an effective control strategy in the U.S.A., through the USDA regulation, between 1990 and 1995 (Hogue and others 1997; Gast and Guard 2011). Due to the fact that *Salmonella* fecal shedding is intermittent, testing this kind of samples does not have reliable results (Gast 2007). Nevertheless, environmental sampling has proven to be relatively easy to perform and the testing sensitivity is high, when the appropriate method is chosen (Arnold and others 2010) although it only indirectly reflects the actual probability of the egg contamination (Gast and Guard 2011). Intensive monitoring for *S.* Enteritidis through the use of drag-swab samples, when sampling from different locations: floors, nest boxes, egg belts, dropping belts, scrapers, fan blades and dust, is considered a very efficient approach and may lead to a high sensitivity detection of *Salmonella* (Davies and Breslin 2001; Kinde and others 2005; Gast 2007). Because many of the *Salmonella* serotypes are invasive, different tissues are often collected and further tested for detecting infected birds (liver, spleen, ovary, oviduct, testes, yolk sac, heart, heart blood, kidney, gall bladder, pancreas, synovia, and eye) (Gast and Beard 1990; Gast 2007). *Salmonella* infections are often a consequence of the pathogen's colonization of the intestinal tract, hence samples of intestinal tissues and contents (ceca and their contents) are often collected for evaluation and in some cases, a low-frequency recovery of *S.* Enteritidis may be possible for long periods of time (Gast and Beard 1990). In the end, egg culturing comes as a confirmatory step in many testing plans, but the detection of *Salmonella* inside eggs is very difficult due to the low incidence at which internal contamination occurs and the very low initial cell densities of salmonellae usually found in freshly laid eggs (Gast 2007). However, in the U.S.A., when an environmental test is positive for *S.* Enteritidis, the FDA Egg Rule (FDA 2009a) requires either continuing the egg testing or diverting eggs to a treatment that will result in at least a 5-log reduction in *S.* Enteritidis. Under the alternative of continuing the egg testing, 4 batches of 1000-egg samples must be tested at 2-wk intervals and if all 4 tests are negative, no further testing is required. Considering the E.U. regulations for laying hens flocks, for all *Salmonella* serotypes with public health significance, in the rearing period, 1-d-old chicks and pullets 2 wk before moving to laying unit must be tested for *Salmonella*, while during laying period, testing must be performed every 15 wk (EC 2003). Furthermore, eggs must not be used for direct human consumption (as table eggs) unless they originate from a commercial flock of laying hens subject to a national *Salmonella* control program.

When a flock has been tested positive for *S.* Enteritidis presence in the environment and the eggs, FDA's egg rule (FDA 2009a) requires that the poultry house in which this flock has resided needs to be cleaned and disinfected through 3 steps: the removal of visible manure, dry cleaning in order to remove dust, feathers and old feed and disinfection with spray, aerosols, fumigation or another appropriate disinfection method (FDA 2009a). During cleaning and disinfection, all the moveable equipment has to be displaced, in order to thoroughly clean all the space. Furthermore, rodent baits have to be placed and removed just prior to cleaning, and in addition, rodent entry sites have to be thoroughly repaired. The sanitation of water lines has to be performed 2 to 3 d prior to

placement of new layers, with water lines filled with an 1870-ppm citric acid solution and a thorough flush of the lines after 2 h. In a 2nd step, the water lines are filled with a 20-ppm chlorine solution and flushed thoroughly after 2 h, without any remaining smell of chlorine (FDA 2009a). The disinfection procedure has to include also an application of the disinfecting solution outside the poultry house, on a 10-feet perimeter.

Poultry facilities are often subjected to disinfection using chemical compounds (especially phenolic and quaternary ammonium ones), following the removal of waste materials and cleaning by high-pressure spraying (Gast 2007). Not only the chemical compounds are different in their efficacy against *Salmonella*. Davison and others (1996) evaluated the differences between 5 classes of disinfectants, with the use of well, stream or pond water, against *S.* Enteritidis. Their results suggested that the inability to remove *S.* Enteritidis from layer houses might in part be associated with the source of water.

Biosecurity is defined as "a program, including the limiting of visitors on the farm and in poultry houses, maintaining personnel and equipment practices that will protect against cross contamination from one poultry house to another, preventing stray poultry, wild birds, cats and other animals from entering the poultry houses, and not allowing employees to keep birds at home, to ensure that there is no introduction or transfer of *S.* Enteritidis onto a farm or among poultry houses" (FDA 2009a).

The current control programs applied in laying hens farms include the following recommendations: (1) to obtain the eggs and chicks only from breeding flocks proven to be *Salmonella* free; (2) to properly disinfect the hatching eggs and that hatching should take place under conditions of stringent sanitation; (3) to clean and disinfect thoroughly the poultry houses between flocks, using recommended procedures; (4) to incorporate rodent- and insect-control measures into the house design and management, and to document their implementation through periodic monitoring; (5) to implement rigidly enforced biosecurity practices, through the restriction of the personnel movement and control of equipment on the poultry-housing premises and between the houses; (6) to ensure that feed is pelleted and does not contain animal proteins; and (7) to ensure the microbiological quality of the water, through treated sources (Gast 2007; FDA 2009a).

The control of people and equipment is considered critical for preventing the introduction of *Salmonella* in the farm, among other pathogens. One of the most effective ways to control human traffic is the use of signs, fences and gates, while buildings should remain locked to the extent possible to ensure that the plans are followed. Nonfarm employees will receive special disposable or reusable clothing or coveralls, after reporting to a central location and signing a logbook before coming on the farm (FDA 2009a).

Sharing equipment is not recommended, but if this happens, it should be ensured that it is clean and disinfected between farms (FDA 2009a). Knape and others (2002) mention 2 general classes of commercial egg processing facilities, in-line and off-line. The in-line type refers to multiple houses connected by a common egg belt, while off-line type refers to eggs coming from houses not connected to the processing plant. Overall, the authors found that aerobic plate counts (APC) of eggs obtained from the in-line type of facility were higher in comparison to those obtained from the off-line one. Also, Musgrove and others (2012) determined the possibility of nest run egg carts to act as reservoir of *Salmonella*, with comparisons between an off-line facility and a mixed-operation one (with in-line processed eggs and supplementation with off-line ones). It appears that *Salmonella* prevalence in the off-line facility (12%) was significantly different ($P < 0.001$) from the mixed-operations one (36%).

It is also necessary to limit the exposure of laying hens to different vectors of this pathogen, such as mice, insects and wild birds, to reduce the risk of *Salmonella* introduction in the flocks (Henzler and Opitz 1992; Davies and Wray 1995; Olsen and Hammack 2000; Mian and others 2002). The FDA Egg Rule (FDA 2009a) mentions that monitoring is essential in the control program of pests. Visual inspection and monitoring by mechanical traps or glueboards is the method to achieve satisfactory rodent control. As for flies, the use of spot cards, scudder grills or sticky traps will help evaluate the level of fly activity and interfere to achieve satisfactory control over them. Furthermore, the harborage of pests must be avoided through removal of debris within and outside the poultry house (FDA 2009a).

Holt and others (2011) consider that the different housing systems may influence the relative effectiveness of the biosecurity measures and the on-farm levels of potential *Salmonella* vectors, thus affecting the success of remediation and prevention methods. The interaction of laying hens with wildlife is increased in free-range housing systems, compared to aviaries or cage systems. In addition, the already contaminated soil can act as a persistent source of *Salmonella*, as it is very difficult to disinfect.

Vaccination

The control of *Salmonella* spp. infection in hen eggs includes various preventive measures, among the most frequently used being vaccination (Van Immerseel and others 2005b).

Active immunization is achieved by inoculation with microbial pathogens that induce immunity but do not cause disease, or with antigenic components extracted from the pathogens. When it is successful, a subsequent exposure to the pathogenic agent elicits an intensified immune response that will eliminate the pathogen or will prevent the disease mediated by its products (Goldsby and others 2003). Many of the common vaccines currently used at a commercial level in poultry consist of inactivated (killed) or live, but attenuated, *Salmonella* spp. strains. Live vaccines generally confer better protection than inactivated ones, the former stimulating both cell-mediated and humoral immunities (Van Immerseel and others 2005b). Live vaccines have been successfully used in the E.U., showing their capacity to reduce the reproductive tract colonization and further on the internal egg contamination risk in laying hens. The vaccine strains were not detected in 1575 eggs from the vaccinated group, hence demonstrating the safety of the approach (Gantois and others 2006).

S. Enteritidis vaccines proved to be efficient in decreasing egg contamination and *S.* Enteritidis and *S.* Typhimurium colonization following challenges (Nakamura and others 1994; Cerquetti and Gherardi 2000a, 2000b; Liu and others 2001; Woodward and others 2002; Khan and others 2003; Van Immerseel and others 2005c). Different studies including *Salmonella* challenges on laying hens followed by vaccine administration proved that the risk of infection with *S.* Enteritidis as well as *S.* Typhimurum can be decreased via this approach (Table 1). Toyota-Hanatani and others (2009) assessed *S.* Enteritidis contamination of eggs laid by vaccinated (inactivated vaccine) and nonvaccinated flocks. More than 1600 *S.* Enteritidis cells/100 mL (most probable number—MPN) of liquid egg samples were isolated from the nonvaccinated flock. For the vaccinated flocks, the maximum value for MPN was 8/100 mL, the risk for a foodborne *S.* Enteritidis outbreak being considerably reduced.

Table 1–Different *in vivo* studies of the effects of *S.* Enteritidis vaccines in poultry.

Immunization way and type of vaccine	*Salmonella* challenge conditions	Observed effects	Reference
Single oral or intramuscular immunization with formalin-inactivated *S.* Enteritidis encapsulated in biodegradable microspheres	10^9 CFU *S.* Enteritidis at 6 wk of age	Decrease of fecal shedding and organ colonization	Liu and others (2001)
Intramuscular immunization with *S.* Enteritidis PT4 bacterin (Salenvac)	5–7.5 $\times$ 10^7 CFU *S.* Enteritidis at 8, 17, 23, 30, and 59 wk	Reduction of the number of tissues and fecal samples that were culture-positiveFewer positive eggs from vaccinated laying hens.	Woodward and others (2002)
Subcutaneous immunization of 9-wk-old chicken with 2 outer membrane proteins of *S.* Enteritidis, followed by 2 booster immunizations with time intervals of 2 wk	8 $\times$ 10^8 CFU of *S.* Enteritidis virulent strain	~1000-fold decrease in cecal colonization	Khan and others (2003)
Oral immunization with 10^9 CFU of a temperature-sensitive mutant of *S.* Enteritidis at 1, 2, 3, and 7 d	10^9 CFU of a virulent *S.* Enteritidis strain at 14 d after the last immunization	Decrease of shedding and colonization of internal organs	Cerquetti and Gherardi (2000a)
Two sets of immunizations, combining oral and intraperitoneal ways with a temperature-sensitive mutant of *S.* Enteritidis	10^8 CFU of *S.* Enteritidis and *S.* Typhimurium at 7 and 14 d after the last vaccination	Fewer bacteria recovered from the cecal contents, liver and spleen 14 d postchallenge	Cerquetti and Gherardi (2000b)
Immunization with oil-emulsion vaccine of a *S.* Enteritidis PT4 strain at 14 and 18 wk of age	10^6 and 10^3 cells of homologous *S.* Enteritidis strain	*S.* Enteritidis isolated from cecal droppings of fewer vaccinated hens	Nakamura and others (1994)

Atterbury and others (2009) aimed to determine the efficacy of a killed *Salmonella* vaccine and 3 live vaccines in preventing cecal colonization of pullets following a challenge with *Salmonella* Enteritidis PT4. There were no significant differences in the total number of positive birds between the groups given the different vaccines following a *Salmonella* challenge. Despite this, the group vaccinated with the killed vaccine contained the smallest number of birds with directly countable levels of *Salmonella* in their ceca ($\geq 10^2$ CFU/g), compared with the unvaccinated group and followed by the live vaccines' groups. The oral administration of a live vaccine to newly hatched chickens results in extensive gut colonization and strong adaptive immunity. In addition, a large presence of bacteria originating from a live *Salmonella* vaccine in the intestine can induce infiltration of polymorphonuclear cells into intestinal walls, conferring resistance to invasion and systemic spread of virulent *Salmonella* strains (Omwandho and Kubota 2010).

Purified-type1 *S.* Enteritidis fimbriae has been used as an antigen in a vaccine and triggered the presence of IgG and IgA in eggs and sera of immunized birds (De Buck and others 2004b). Intravenous vaccination of chickens with a *fimD* mutant of *S.* Enteritidis led to a lower contamination of eggs (De Buck and others 2004a). Immunization with vaccines containing outer membrane proteins (OMP) of *S.* Enteritidis also led to reduced intestinal mucosa colonization in laying hens (Khan and others 2003).

E.U. legislation provides different requirements for food industry stakeholders to apply, in order to reduce the risk of eggs contamination by *S.* Enteritidis. For this purpose, each E.U. country has implemented its own approved national control program, vaccination being one of the adopted measures. The national control programs aim to reduce the prevalence of the foodborne *Salmonella* serotypes. For all *Salmonella* serotypes with public health significance, sampling must be performed at specific moments during breeding and production phases. If the animals are vaccinated, immunological testing would not be performed. Also, "[. . .] eggs originating from flocks with unknown health status, suspected of being infected or being infected may be used for human consumption only if treated in a manner that guarantees the elimination of *Salmonella* serotypes with public health significance" (EC 2003). Through an amendment that modifies Regulation EC 2160/2003 "the eggs originating from flocks with unknown health status and suspected of or being infected with a serotype of *Salmonella* for which it has already been established a point of reduction or being the cause of a foodborne outbreak" are considered as eggs included automatically in class B (EC 2007b). As defined by the Commission Regulation EC no. 557/2007, class A eggs are those that meet the following characteristics: (a) shell and cuticle: normal shape, clean and undamaged; (b) air space: height not exceeding 6 mm, stationary and 4 mm for eggs marketed as "extra"; (c) yolk: visible on candling as a shadow only, without clearly discernible outline, slightly mobile upon turning the egg and returning to a central position; (d) white: clear translucent; (e) germ: imperceptible development; (f) foreign matter; and (g) foreign smell: not permissible. Class A eggs which no longer have the mentioned characteristics, may be downgraded to class B (EC 2007a)

They are marked as such and their introduction in packaging centers depends on the efficacy of applied methods for the prevention of a potential cross-contamination of the eggs coming from healthy flocks.

Vaccination is a mandatory measure used to fight against *Salmonella* in Austria, Belgium (for *S.* Enteritidis), The Czech Republic, Germany, and Hungary, while allowed and recommended in Bulgaria, Belgium (for *S.* Typhimurium) Cyprus, Estonia, France, Greece, Italy, Latvia, Lithuania, The Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, and the United Kingdom (EFSA 2004; EFSA 2011). Denmark, Finland, Sweden, and Ireland have banned vaccination and developed efficient control programs for *Salmonella* spp. They rejected the use of vaccines also due to their potential interference with the results for serological tests (Murchie and others 2007; Kornschober and others 2009). As an example, in Sweden, the national *Salmonella* spp. control program aims to reduce the prevalence of the bacterium by implementing different measures such as the quarantine of all imported poultry and the destruction of any imported birds that would be tested positive for any *Salmonella* spp. Sweden also uses sampling procedures according to E.U. legislation requirements, and the flocks are rapidly depopulated if positive. Also, any traced products on the market are immediately withdrawn. Additionally, there exists a specific control program for *Salmonella* spp. in feed production (Keery 2010).

Salmonellosis is not caused by *S.* Enteritidis or *S.* Typhimurium in Australia, these serotypes being reported as absent in layer flocks. Therefore, the application of a national control program against *Salmonella* has been considered not necessary.

In the U.S.A. (FDA 2009a) and Canada (Keery 2010) the use of vaccines to increase the resistance of birds against *Salmonella* spp. is encouraged. In Canada, the vaccination of the layer flocks introduced into a new house becomes highly recommended of the former flock was tested *S.* Enteritidis-positive (Health Canada 2011).

Passive immunization

Laying hens immunized with antigens from selected microorganisms (for example, *S.* Enteritidis and *S.* Typhimurium) react by producing high quantities of specific antibodies (IgY) which are transported from the blood into the egg yolk. These eggs containing high levels of antigen-specific IgY, called hyperimmune eggs, can be administered as a feed additive (usually in the form of whole yolk powder) to other species to provide them with passive immunity (Chalghoumi and others 2009a). Chalghoumi and others (2008) have shown that it is possible to produce IgY against *S.* Enteritidis and *S.* Typhimurium OMP in the same egg with concentrations of 429 ± 20 mg/g. Moreover, they demonstrated (Chalghoumi and others 2009b) that these specific antibodies have a growth inhibitory effect on *Salmonella* spp., in a concentration-dependent manner. They also assessed the ability of preventing adhesion of *Salmonella* spp. to intestinal cells by using human epithelial Caco-2 cell lines. The results demonstrated that specific IgY was able to reduce the decrease in transepithelial electrical resistance of the infected Caco-2 cell monolayers, blocking adhesion of *Salmonella* spp. in a concentration-dependent manner. Another study demonstrated that orally administered egg yolk antibodies induced a reduction of 13.3% of *Salmonella*-positive eggs (in an experimentally infected group), in comparison to 29.4% *Salmonella*-positive eggs in the group that did not receive the egg yolk powder. The antibodies were administered as a feed additive (3 g/hen/day), in the form of whole egg powder (Gürtler and others 2004).

The risk of the development of resistance phenomenon against these antibodies is highly limited (Xu and others 2011). Indeed, these are polyclonal antibodies, targeting multiple epitopes. Nevertheless, it has been pointed out that resistance phenomena can occur concerning vaccines (Sirsat and others 2009) and this is still a possibility concerning passive immunization even if weak, in our opinion. Sirsat and others (2009) also mentioned the risk of developing too specific tools with antibody therapy or vaccination, which could be particularly problematic for *Salmonella* spp. given the wide range of immunogenic serotypes. However, Chalghoumi and others (2008) observed a high cross-reactivity of anti-*S.* Enteritidis IgY with *S.* Typhimurium (ST)-antigen and *vice versa*. In this experiment, they used indeed OMP as vaccinal antigens; and both *Salmonella* spp. share common epitopes on OMP. Using antigens shared between several serotypes allow, thus, to cope with the risk of a too high specificity of the developed antibodies. Moreover, by developing IgY against 2 *Salmonella* serotypes in the same egg yolk, Chalghoumi and others (2008) left the door open for the further development of hyperimmune eggs targeting a diverse set of pathogens.

Natural antimicrobial products

Bacteriophages. Bacteriophages are bacterial viruses with the ability of using the bacterial cell to multiply. The mechanism of the infectious cycle is used to differentiate the 2 main types of bacteriophages: the virulent ones (that determine lysis and death of the cell in a very short time) and the temperate ones (the latent, using lysogeny in their infectious cycle). Virulent bacteriophages are used in different products destined to reduce foodborne pathogens in foods (Monk and others 2010). Bacteriophages are highly discriminatory, most of them interacting with a specific set of bacteria that express specific binding sites (Joerger 2003).

Using a combination of 3 different *Salmonella*-specific bacteriophages to reduce *S.* Enteritidis colonization in the ceca of laying hens resulted in a significant reduction of the incidence, up to 80%. The cocktail of the 3 bacteriophages was administered via spray at 6 d of age, using a multiplicity of infection of $10^{(3)}$ plaque-forming units. The birds were experimentally infected by oral inoculation with 2.95×10^5 CFU/mL of *S.* Enteritidis, at 7 d of age. At 14 d, *S.* Enteritidis counts in the ceca of control group hens reached 1.56×10^5 CFU/g, while for the bacteriophage-treated group only 9.48×10^3 CFU/g (Borie and others 2009) (Table 2). Additionally, the same study assessed the ability of a combination between a bacteriophage and a competitive exclusion product to reduce cecal colonization by *S.* Enteritidis. The results revealed that this combination treatment was even more efficient (1.6×10^2 CFU/g), compared to the use of bacteriophages alone.

Toro and others (2005) also showed that the use of a "cocktail" of bacteriophages, in a dose of 5.4×10^6 PFU/0.5 mL/bird, on White Leghorn chickens challenged at day 7 with *S.* Typhimurium (suspension of 0.5 mL with 2.4×10^5 CFU/mL) determined a decrease of ileum colonization with this pathogen. The ileum samples collected from bacteriophage-treated chickens showed significantly lower *S.* Typhimurium counts (1.1 CFU/mL) than challenged and untreated ones (81.8 CFU/mL) (Table 2).

Tailspike protein of bacteriophages is a component of the tail apparatus with the role of mediating the specific recognition of its bacterial host by binding to its surface structures. After oral administration to 1-d-old Leghorn chickens, it resulted in a significant delay of *Salmonella* spp. growth and colonization and a significant reduction of *Salmonella* spp. counts at the level of the ceca, liver, and spleen, in comparison with control groups (Waseh and others 2010). According to these authors, their efficacy depends on their degree of resistance to gastrointestinal proteases.

Generally regarded as safe, bacteriophages are considered a highly efficient tool for the biocontrol of pathogens in food products (Garcia and others 2008). Phage therapy can be successfully applied to reduce the *S.* Enteritidis level on poultry carcasses after slaughter (Higgins and others 2005a, 1997). In spite of their high specificity, bacterial resistance has been encountered. Resistance to bacteriophages occurs when losses or modifications of bacterial cell surface molecules (like the LPS, pili, or flagella) take place, those being used usually as receptors (Levin and Bull 2004). However, bacteriophages possess the ability to change rapidly in response to the emergence of bacteria-resistant mutants (Sulakvelidze and others 2001; Carvalho and others 2012). Therefore, the use of different bacteriophages in what is called a "cocktail" has been found necessary even to fight against a single bacterial strain (Joerger 2003).

Protein and fiber sources. Kassaify and Mine (2004a) demonstrated that nonimmunized egg yolk powder could suppress the colonization of *S.* Typhimurium in laying hens (Table 2). The nonimmunized egg yolk powder was prepared as it follows: the eggs were cracked after disinfection of the exterior shell surface and the egg yolks were aseptically separated from the albumen; the pooled yolks were freeze-dried and crushed into a fine

Table 2–Different *in vivo* and *in vitro* studies concerning natural antimicrobials on laying hens and broilers for reduction of *S.* Enteritidis or *S.* Typhimurium colonization in different organs and tissues.

Type of natural antimicrobial used	*Salmonella* challenge conditions	Observed effects	Reference
A cocktail of 3 bacteriophages administered via spray at 6 d of age, at a dose of 10^3 plaque forming units	Oral inoculation with 2.95×10^5 CFU/mL of *S.* Enteritidis at 7 d of age	Cecal counts drop to 9.48×10^3 CFU/g, in comparison to 1.56×10^5 CFU/g for the control group	Borie and others (2009)
Oral administration of 3 doses of tailspike proteins of bacteriophages with 10% BSA[a] at 1-d-old age in 2 protocols: at 1 h, 18 h, and 42 h (Protocol 1) and at 18 h, 42 h, and 66 h (Protocol 2)	Oral gavage with 10^4–10^7 *Salmonella*/ 300 μL PBS[b] at 2 d of age	Significant reduction of *Salmonella* in the ceca, liver, and spleen in both protocols	Waseh and others (2010)
A cocktail of bacteriophages in a dose of 5.4×10^5 CFU/mL/bird at the age of 1 d	Oral administration of a suspension containing 2.4×10^5 CFU/mL of *S.* Typhimurium at day 7	Decrease of *S.* Typhimurium counts in the ileum	Toro and others (2005)
Feed supplemented with nonimmunized egg yolk powder at 10% concentration (wt/wt) for 4 wk, beginning at the age of 1 d	Oral administration of 1.0 mL of 10^9 CFU/mL *S.* Typhimurium 2 times at the end of the 4-wk period	No detection of *S.* Typhimurium in any organs from the egg yolk powder treated group; 3.1 to 4.0 $\log_{10}$ cfu in the sampled organs (intestine, ovary, and oviduct) of the positive control group.	Kassaify and Mine (2004a)
90% of alfalfa and 10% basal diet (1), 90% alfalfa + 10% basal diet with 0.375% FOS (2) and 90% alfalfa + 10% basal diet with 0.75% FOS (3) at the age of 1 d	Crop gavage on day 4 with 1 mL of inoculum containing 10^5 CFU of *S.* Enteritidis phage type 13a	Reduction of ovary and spleen colonization by *S.* Enteritidis for FOS- containing diets' method when compared to feed withdrawal	Donalson and others (2008a)
Product containing10^{10} viable spores of *B. cereus* var. *toyoi* (powder feed additive) included at 0, 20 or 100 mg/kg of feed at the age of 1 d	Oral suspension containing 2×10^8 CFU *S.* Enteritidis/mL administered at day 7	3 wk p.i. 38% of the treated birds positive for *S.* Enteritidis (63% in the control group)	Vilà and others (2009)
1 g of *Saccharomyces boulardii*/kg feed (trial 1) and 100 g of the same product/kg feed (trial 2) administered to 1-d-old broiler chickens	Oral gavage on day 4 with 3.3×10^8 *S.* Typhimurium	Reduction of *S.* Typhimurium colonization frequency from 70% in the control group to 20% and 5% in trial 1 and trial 2, respectively	Line and others (1998)
Acidifier in a dose of 1.5 kg/t of feed (A) or 3.0 kg/t of feed (B) on 1-d-old chicken	Crop inoculation at 3-d-old with 0.1 mL of *S.* Enteritidis suspension containing $1.3 - 3.3 \times 10^9$ CFU/mL.	Reduction to 3.47 log CFU/g *S.* Enteritidis in cecal contents for dose B and 4.59 log CFU/g *S.* Enteritidis for dose A, compared to 5.06 log CFU/g *S.* Enteritidis in control group	Sterzo and others (2007)
Aromatic product composed of eugenol (250 ppm) in an amorphous SiO_2 inert carrier, administered as feed additive 1st at 15-wk-old, during a 3-wk period	Inoculation into the crop of 1 mL (single dose), containing 3.2×10^7 CFU *S.* Enteritidis at 18 wk of age	Isolation of *S.* Enteritidis after 2 wk p.i. in feces and 3 wk p.i. in eggs; positive results in liver, spleen, and ovary 15 d p.i. but negative at 29 d p.i.	Ordoñez and others (2008)
Dietary capsaicin at doses of 18 ppm (A) and 36 ppm (B) administered in feed, for 28 d, beginning at the age of 1 d	Challenge on day 27 with 10^8 CFU/mL of *S.* Enteritidis	Reduction of *S.* Enteritidis colonization in liver and spleen for both groups (A – 56.67%; B – 43.33%); control group (feed without capsaicin) – 76.67%.	Vicente and others (2007)

[a] Bovine serum albumin.
[b] Phosphate buffer solution.
p.i. = postinoculation.

powder. Following infection with 10^9 CFU of *S.* Typhimurium per bird, the addition of 5.0, 7.5, and 10.0% (wt/wt) nonimmunized egg yolk powder to the feed rapidly decreased the number of *S.* Typhimurium in feces samples. Indeed, the counts reached 10% of the initial values, after only a week, with a significant difference in comparison to the positive control group (nonsupplemented feed). Moreover, after 2 wk of feeding egg yolk powder at a dose of 10.0%, *Salmonella* was completely undetected.

In another research study, Kassaify and Mine (2004b) demonstrated that at a concentration as low as 5% (wt/wt) in the feed, nonimmunized egg yolk powder eliminated *S.* Enteritidis at the intestinal level after the 1st week, demonstrated by the negative results obtained for the tested fecal samples.

This may be explained by the presence of components such as high-density lipoproteins (Kassaify and others 2005) or sialyloligosaccharides and their derivatives (Sugita-Konishi and others 2002).

Competitive exclusion flora, probiotics, prebiotics, and organic acids. The use of competitive exclusion flora, probiotics, prebiotics, as well as acid-based products, have been widely investigated worldwide as preventive methods for *Salmonella* spp. colonization in poultry (Seo and others 2000; Van Immerseel and others 2002; Wagner and Cerniglia 2005; Donalson and others 2008b; Dunkley and others 2009).

Competitive exclusion products involve the introduction of intestinal bacteria from mature healthy poultry to newly hatched chickens, the concept being defined as "the early establishment of an adult intestinal microflora to prevent subsequent colonization by enteropathogens." The mechanism used by the bacterial species from the competitive exclusion products to inhibit the proliferation of other bacteria consists of creation of a restrictive physiological environment (for example, bacteriostatic effect of VFA in the ceca). Added to this are the following: competition for bacterial receptor sites, elaboration of antibiotic-like substances (such as bacteriocins), and depletion of or competition for essential substrates (Schneitz and Mead 2000; van der Wielen and others 2000; Joerger 2003; Callaway and others 2008).

Competitive exclusion products represent a mix of different bacterial species, usually derived from cecal contents and/or wall of healthy domestic fowl. Several products have been evaluated as

competitive exclusion cultures. Schneitz (2005) and Schneitz and Mead (2002) mention a wide variety of such products. Several of them consist of unrefined mixed cultures of whole cecal contents from adult chickens, while others contain selected mixed cultures, with addition of cecal walls scrapings, including identified genera of bacteria, efficient against *S.* Enteritidis and *S.* Typhimurium cecal colonization. Because most of them include a mix of not entirely determined bacteria, isolated from the avian gut, and in spite of their proved effectiveness, the incertitude concerning their composition is reducing the rate of their use in the egg and broiler meat production sector (Van Immerseel and others 2005b).

A probiotic is defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller 1989). A variety of microbial species have been used as probiotics, including species of *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Streptococcus* as well as a variety of yeasts (Patterson and Burkholder 2003). The potential mechanisms that allow the exclusion of pathogenic species, among them *S.* Enteritidis, by the probiotics include competition for adhesion sites and nutrients or production of antimicrobial compounds, such as bacteriocins, VFA, or hydrogen peroxide (Erwing 2009; Vandeplas and others 2010). Besides the inhibition of cecal colonization by pathogens, it has been demonstrated that probiotic bacteria determined an increase of the oxidative burst capacity and degranulation of heterophils isolated from chicks 24 h after probiotic administration. This suggests a possible activation of the innate immune system (Farnell and others 2006).

In poultry (laying hens and broilers), bacteria of the genus *Lactobacillus* have been frequently studied for its effects on reducing or inhibiting *Salmonella* cecal colonization (Gusils and others 1999; Pascual and others 1999; Jin and others 2000; Tellez and others 2001; Ammor and others 2007; Lima and others 2007). It has been suggested that lactobacilli isolated from either cloaca or vagina of laying hens present *in vitro* inhibitory activity against *S.* Enteritidis, with no differences noticed between those isolated from the cloaca and the ones from the vagina (Miyamoto and others 2000). Van Coillie and others (2007) have also demonstrated that lactobacilli isolated from the cloaca and the vagina of laying hens inhibited *Salmonella* growth *in vitro* and decreased *S.* Enteritidis colonization *in vivo*. *Salmonella* inhibition was shown to depend on the species of *Lactobacillus*, correlated to some extent with the production of lactic acid of each.

Another probiotic with potential use in laying hens is based on an active ingredient consisting of *Bacillus cereus* var. *toyoi* spores (EC 2001; Tellez and others 2012). Its efficacy against *S.* Enteritidis has been demonstrated on poultry by Vilà and others (2009) (Table 2). They challenged laying hens with a *S.* Enteritidis suspension containing 2×10^6 CFU/mL for trial 1 and 2×10^8 CFU/mL for trial 2, respectively. The product containing 10^{10} viable spores was administered as feed additive in concentrations of 20 mg/kg (trial 1) and 100 mg/kg (trial 2). The results showed that *S.* Enteritidis was not detected in the probiotic-administered groups, while for the control group, 42% of the birds were positive. These authors suggested that the product encourages the proliferation of *Lactobacillus* spp., improving the balance of the intestinal microflora.

Yeasts were also studied for their potential to act as probiotics in poultry, against pathogens like *Salmonella* spp. Line and others (1998) showed that including *Saccharomyces boulardii* reduced the frequency of cecal colonization from 70% in the positive control broilers to 20% and 5%, respectively (2 trials) (Table 2). The possible mechanism to reduce the cecal colonization of such pathogens could be the possibility for the yeast to act as a pathogen-adherent microflora, due to the mannose content of *Saccharomyces boulardii*'s cell wall, with potentially successful use in egg production sector. Another mechanism was suggested by Pontier-Bres and others (2012) who revealed that *Saccharomyces boulardii* was able to modify *S.* Typhimurium motility and trajectory, registering a decrease in its invasion properties.

Another option as a preventive method is the use of prebiotics. They can be regarded as an integrated approach to an improvement of food safety, starting with the maintenance of a healthy intestinal ecosystem (Gaggia and others 2011). Among the beneficial effects of prebiotics these can be mentioned: stimulation of the immune system, reduction of inflammatory reactions, toxin inactivation, modification of the intestinal microbiota, increased production of VFA, and prevention of pathogen colonization (Patterson and Burkholder 2003; Revolledo and others 2006; Salminen and others 2010).

Prebiotics are not digested or metabolized, or they are metabolized very little, during their passage through the upper portion of the gastrointestinal tract (GIT). Therefore, they enter the ceca without any change to structure, being fermented by the colonic flora. Through the stimulation of bifidobacteria, they may have the ability to inhibit pathogenic bacteria such as *Salmonella* spp. (Grizard and Barthomeuf 1999; Doyle and Erickson 2006; Vandeplas and others 2010). Lactose (Ziprin and others 1993), fructooligosaccharides (FOS) (Fukata and others 1999), mannanoligosaccharides (MOS) (Fernandez and others 2002), and isomaltooligosaccharides (Chung and Day 2004) are highly effective prebiotics already applied in the broiler chicken industry for the inhibition of *Salmonella* spp. cecal colonization. It has been shown that FOS are highly effective in reducing chicken intestinal colonization by *Salmonella* spp., by exerting a preferential stimulatory effect on several bacteria of 2 health-promoting genera (*Bifidobacterium* spp. and *Lactobacillus* spp.), while maintaining populations of potential pathogens at relatively low levels (Xu and others 2003). During an *in vitro* fermentation test, cecal contents collected from laying hens were diluted to a 1:3000 concentration with an anaerobic dilution solution and added to serum tubes filled with ground alfalfa or a layer ration with or without FOS, the latter as a prebiotic. The concentrations of VFA and lactic acid were quantified at 6 and 24 h of substrate fermentation. The results showed a greater production of VFA and lactic acid compared with the layer ration. The amendment of FOS to both alfalfa and the layer ration appeared to further increase fermentation, with a more pronounced effect after 24 h of fermentation (Donalson and others 2008a).

Kaplan and Hutkins (2000) also showed that different species of *Lactobacillus* (*L. bulgaricus*, *L. acidophilus*, *L. plantarum*, *L. casei*) as well as *Bifidobacterium* (*B. adolescentis*, *B. breve*, *B. infantis*, *B. longum*) were able to use FOS in a FOS-MRS broth. Fernandez and others (2002) showed that laying hens' diets supplemented with MOS resulted in a decrease in S. Enteritidis hen cecal contents over time, by increasing the Bifidobacterium spp. and *Lactobacillus* spp. levels.

However, more recent studies have focused on investigating prebiotics' efficacy during the molting period, as this practice has been proven to increase to some extent laying hens' susceptibility to *Salmonella* infections (Donalson and others 2008b) (Table 2). Adding FOS to a combination of 90% alfalfa and 10% layer ration, in 2 levels (0.750% and 0.375%) resulted in a reduced ovary and liver colonization by *S.* Enteritidis, while the counts decreased significantly. It appears that between the 2 doses used for FOS administration, the authors consider as sufficient the lower one.

Due to the ability of prebiotics to stimulate some beneficial microflora populations such as bifidobacteria (Šuškovic and others 2001; Vandeplas and others 2010) another approach was developed. Synbiotics are combinations of probiotic strains and prebiotic substrates, their use being regarded as a way of stabilization and/or improvement of the probiotic effect. The combination is thought to be able to improve the survival of the probiotic microorganism, because its specific substrate is readily available for its fermentation (Collins and Gibson 1999). Nisbet and others (1993) showed that by using a combination of mixed cecal microflora continuous flow culture a reduction of 1.75 $\log_{10}$ *S.* Typhimurium colonization in chicken was observed; by using 5% dietary lactose as a prebiotic, the decrease was 2.98 $\log_{10}$. A combination of the 2 succeeded in reducing the cecal colonization by 4.27 $\log_{10}$, suggesting the success of these combined approaches.

It is also possible to decrease egg contamination risk by adding organic acids to the feed or drinking water at an appropriate time (Thompson and Hinton 1997). Van Immerseel and others (2007) have extensively described the mechanisms involved in organic acids' activity. The effect of feed with added organic acids (commercial blend) in a quantity of 3 kg/ton of feed represented an efficacious and cheap prevention method for cecal colonization by *S.* Enteritidis in a challenge experiment with laying hens (Sterzo and others 2007) (Table 2).

Butyric acid is the most frequently used organic acid as a feed or drinking water additive. The efficacy of butyrate in powdered form and sodium salt of n-butyric acid (30%) in microencapsulated (coated) form was assessed 3 d after inoculation with *S.* Enteritidis, during 2 trials that included young layer chickens and broilers. The studied parameters were cecal and internal organ colonizations. The results showed that coated butyric acid was superior to uncoated butyric acid in reducing *S.* Enteritidis colonization on both trials (Van Immerseel and others 2005a, 2007). Foster (2001) showed *in vitro* that organic acids determine acid resistance in *Salmonella* spp. through a complex process of pH homeostasis induction. Indeed, it had already been shown that for *S.* Typhimurium the exposure to short-chain fatty acids (SCFA) increased to some extent the virulence of this pathogen by increasing its acid resistance (Kwon and Ricke 1998; Sirsat and others 2009). The induction of an acid tolerance response involves growth of the acid-sensitive microorganisms in a moderately low pH environment. This subsequently leads to survival when the species is suddenly exposed to what would normally be considered lethal acidic conditions, thus protecting *Salmonella* spp. against the effects of organic acids (Ricke 2003). Van Immerseel and others (2006) also showed that SCFA can regulate the invasive phenotype of *Salmonella* spp., and that preincubation of *Salmonella* with SCFA increased acid resistance and survival in macrophages. The same authors mentioned that medium-chain fatty acids possess even greater bacterial activity against *Salmonella* spp. than the SCFA. Considering the latter, Durant and others (2000) assessed the expression of 2 transcriptional regulators of SPI-1, *hil*A, and *inv*F, needed for host tissue invasions. Growth rates of *Salmonella* spp. were reduced by increasing the SCFA concentrations at pH 6, but not the same happened at pH 7. The pH-dependent manner of induction suggested that entry of SCFA into the host cells is necessary; these fatty acids possibly serve as an environmental signal that triggers the expression of invasion genes in poultry GIT.

Essential oils. Many studies performed until now on the possibility of using essential oil (EO) as active antimicrobial ingredient in animal feed *in vivo* or *in vitro*, showed high variability in the results obtained. An overview on the potential of EOs in poultry production and their possible modes of action, among them antimicrobial activity, has recently been published by Brenes and Roura (2010).

An EO is "a mixture of fragrant, volatile compounds, named after the aromatic characteristics of plant materials from which they can be isolated" (Lee and others 2004). Being already known that EOs are more active against Gram-positive bacteria, in comparison to Gram-negative ones, it has been shown *in vitro* that cinnamaldehyde (obtained from cinnamon) moderately inhibits *L. acidophilus* and *B. longum* (Lee and Ahn 1998). This could suggest the existence of an undesired effect on the gastrointestinal microflora. However, Lee and others (2004) suggested that the selective inhibition shown by the cinnamaldehyde on pathogenic intestinal bacteria may have a pharmacological role in balancing the intestinal microflora. In addition, Ouwehand and others (2010) showed that potentially beneficial bacteria (such as *Bifidobacterium* spp.) are resistant or only a little susceptible to the majority of EOs tested *in vitro*. On the contrary, these authors proved that *L. fermentum* or *B. breve* were stimulated in their growth by several EOs (Ouwehand and others 2010).

Helander and others (1998), and afterwards Cosentino and others (1999), evaluated the minimum inhibitory concentrations (ppm) for 3 different EOs against *S.* Typhimurium during *in vitro* studies. Carvacrol (obtained from oregano and thyme) showed values of 150 and 225 ppm, respectively, cinnamaldehyde showed a level of 396 ppm (with no value offered by Cosentino and others 1999) and thymol (obtained from common thyme) showed values of 150 and 56 ppm, respectively. O'Bryan and others (2008) reported the antimicrobial activity of orange EOs against *Salmonella* spp. (including *S.* Enteritidis and *S.* Typhimurium), using the disc diffusion assay. The most effective orange EOs were composed mainly of d-limonene (94%) and myrcene (about 3%). Moreover, Johny and others (2008) have shown *in vitro* efficacy of trans-cinnamaldehyde, a safe ingredient obtained from cinnamon, against *S.* Enteritidis that could possibly be added to chicken drinking water. Chao and others (2000) showed that the essential oils extracted from cinnamon were effective against both Gram-positive and Gram-negative bacteria. Ouwehand and others (2010) showed the same. They evaluated 13 different essential oils for the ability to inhibit the growth *in vitro* of several bacteria species, among them *S.* Infantis, *S.* Enteritidis, and *S.* Typhimurium. All 3 serotypes were significantly inhibited when using the maximum level of EOs (500 mg/L). The most effective EOs against these were carvacrol, cinnamaldehyde, citral, and thymol.

Ordoñez and others (2008) concluded that, in commercial production layers, eugenol (obtained from clove) seems to aid in the cleaning of intestinal and systemic infections, thus playing an important role in the control of *S.* Enteritidis cross-contamination of table eggs. Finally, Vicente and others (2007) observed in laying hens the prophylactic effect of capsaicin (obtained from chili peppers) in an experimental *S.* Enteritidis infection (Table 2).

The efficacy of EOs against foodborne pathogens depends on the active molecule, on the targeted pathogen, but it must be mentioned that the chemical composition of EOs can greatly vary for a particular plant species. This depends on the part of the plant, the geographic origin, and the harvesting period in a year or even in a day. This may be a possible explanation for the variability of the results obtained from one experiment to another. Moreover, whole EOs can have greater antimicrobial activity than their major isolated constituents can. As an example, antimicrobial activity of allyl sulfur compounds of garlic oil was shown to increase with

each additional sulfur atom, suggesting that the effect is a result of synergy among the different compounds. Therefore, the antimicrobial activity of garlic oil may be more powerful than the activity of its main compounds individually (Calsamiglia and others 2007).

The possibility for a foodborne pathogen to develop resistance to EOs exists. Ultee and others (2000) observed a decrease of sensitivity of *B. cereus* toward carvacrol. The cause of this decrease was believed to be the growth of this pathogen in the presence of nonlethal carvacrol concentrations. However, the risk of resistance development against EOs is still extremely rare (Sirsat and others 2009).

Considering the possibility of a change in odor and palatability of the EO-supplemented rations, Windisch and others (2008) suggested that the use of phytogenic feed additives, a series of botanicals already containing EOs, improved feed flavor and enhanced hen production performance. This leads to a potential conclusion that EOs may modify in a positive way the organoleptic characteristics of feed, therefore no decreases in ingestion should occur.

Bacteriocins. Bacteriocins are proteins, produced by some bacteria, that act against other closely related bacteria. The family of bacteriocins includes a large diversity of proteins differing in size, the microbial target, and mode of action. Two main groups can be distinguished: those produced by Gram-positive bacteria and the ones produced by Gram-negative bacteria (Gordon and others 2007; Heng and others 2007).

Most of the bacteriocins differ from classical antibiotics through their ribosomal origin and their great specificity (Riley and Wertz 2002a, 2002b). Bacteriocins may possess a bactericidal or bacteriostatic mode of action on sensitive pathogens, depending on the dose and degree of purification, physiological state of pathogen (growth phase), and experimental conditions. The majority of bacteriocins uses membrane permeabilization or interferes with essential enzymes to cause cell death (Peschel and Sahl 2006; Pithva and others 2011). For example, nisin forms a complex with ultimate cell wall precursor lipid II, a hydrophobic carrier of peptidoglycan monomers (Dias Paiva and others 2011). Further, it uses this compound as a docking molecule for its pore-forming activity, leading to the inhibition of bacterial cell wall biosynthesis. In addition, nisin is able to induce a rapid efflux of ions or cytoplasmic solutes such as amino acids and nucleotides. The concomitant depolarization of the cytoplasmic membrane determines an instant termination of all biosynthetic processes (Wiedemann and others 2001).

As antimicrobials, bacteriocins may be used as food preservatives or feed additives. Hereafter, we will develop only the feed additive aspects.

Bacteriocins are often considered more natural in contrast to antibiotics, as they are thought to have been present in many of the foods eaten since ancient times (Cleveland and others 2001). Also, bacteriocins are produced by lactic acid bacteria, which have been demonstrated to be beneficial for human health (Joerger 2003). Therefore, they can be used without risk in food-producing animals, their application being consistent with consumer demand for natural food products.

Gram-negative bacteria are less sensitive to bacteriocins than the Gram-positives. However, microcins—bacteriocins produced by *Escherichia coli* strains—which are smaller than colicins possess the capacity to inhibit Gram-negative bacteria (Diez-Gonzalez 2007). Microcin J25, for example, is active against *Salmonella* spp., including *S.* Enteritidis (Portrait and others 1999). Microcin J25 is highly resistant to digestive proteases and could affect the gastrointestinal microbiota, when ingested in feed (Galvez and others 2010). On the other hand, a chymotrypsin-susceptible microcin J25 variant may be used as a food preservative (Pomares and others 2009) in combination with other hurdles. Line and others (2008) demonstrated *in vitro* (the spot test) strong antibacterial properties of a bacteriocin produced by enterococci (an enterocin)—E-760—against a broad spectrum of foodborne Gram-negative or Gram-positive pathogens, including *S.* Enteritidis and *S.* Typhimurium. Svetoch and others (2008) observed that oral administration of bacteriocins E 50-52 in chicken feed resulted in a significant reduction of *S.* Enteritidis, at the cecal level, but also in the liver and the spleen, while enumeration of lactic acid bacteria was not significantly different in the ceca of both treated and untreated groups. Therefore, it is expected that the results obtained for chickens could be applied to laying hens also.

The modes of action of bacteriocins are complex and not fully understood. Most of them act in different ways to inhibit or kill sensitive bacteria (Rossi and others 2008). It is the reason why development of pathogen strains which manifest resistance to antimicrobial peptides has been considered difficult, if not impossible (Hancock and Chapple 1999; van't Hof and others 2001). However, Lin (2009) and Sirsat and others (2009) mentioned the factual resistance to bacteriocins (nisin) through the altering of target bacterial cell surface receptors.

In order to reduce the cost of bacteriocins to a price that any producer could afford, Lin (2009) considers necessary an improvement in the production process. In addition, Gaggia and others (2011) suggested that the use of bacteriocins-producing lactic acid bacteria would have more advantages, considering the cost also, than using purified bacteriocins alone. The application of pure bacteriocins in food may present reduced efficacy, as they could bind to food components.

Despite the promising results obtained with the use of bacteriocins, more research on combinations of bacteriocins or on associations between bacteriocins and other treatments is needed.

Postharvest Methods for Reducing the Risk of Salmonellosis Due to Contaminated Shell Eggs Consumption

Shell eggs storage and prevention of growth and multiplication of *Salmonella*

Prompt refrigeration to temperatures capable of restricting microbial growth has been recommended as an approach to reducing the likelihood that contaminated eggs will transmit *S.* Enteritidis to humans (Gast and Holt 2000).

In 2000, FDA published a final rule in the Federal Register (65 FR 76092), which states that a proposed maximum ambient temperature of 7.2 °C (45 °F) would extend the effectiveness of the egg's natural defenses against *S.* Enteritidis and would slow the growth rate of this foodborne pathogen (FDA 2000). In the Final Egg Rule (FDA 2009a), this proposition is maintained, as it is specified that this maximum value needs to be applied not only during storage, but also during transport, beginning 36 h after the time of lay. As an exception, shell eggs may be stored at ambient temperature values (above 7.2° C) if they are directed to a following step of processing, but not for more than 36 h. However, refrigeration must be kept even when using ionizing radiation (which results in only 2 to 3 logs reduction of *S.* Enteritidis), as this procedure is not regarded as efficient as the use of pasteurization (which ensures a 5 log reduction of *S.* Enteritidis) (FDA 2009b).

In Canada, shell eggs and those sent to a processing station must be kept under refrigeration, or stored for a maximum of 6 d at storage temperatures of 20 °C or less, or stored for a maximum

of 2 d at temperatures between 20 °C and 30 °C (Health Canada 2011).

Concerning cold storage of eggs in the E.U., EC Commission Regulation 589/2008 specifies that "[. . .] eggs should be stored and transported at a constant temperature, and should in general not be refrigerated before sale to the final consumer [. . .]" (EC 2008). Nevertheless, it has been shown that *Salmonella* spp. growth inside the egg is influenced by storage temperature.

Research in this field has proved that ambient temperatures are not proper for the storage of shell eggs, especially since the risk of *S.* Enteritidis horizontal transmission has increased, and further on, due to its capacity of growth and multiplication inside the shell eggs. Martelli and Davies (2012) suggested that the temperature values for shell eggs storage should not exceed 20 °C. In egg albumen, *Salmonella* spp. can grow at 20 °C, while unable to grow at temperatures below 10 ° C, therefore showing that a temperature value for optimal storage of eggs should not exceed this last value.

Foodborne pathogens such as *S.* Enteritidis can grow in the contents of naturally contaminated eggs at room temperature (20 °C) and it does not lead to changes in the color, smell and consistency of the egg contents. However, the multiplication of *S.* Enteritidis in the stored eggs appears to be associated with alteration of the yolk membrane, which allowed the bacterium to either invade the yolk or obtain nutrients from it (Humphrey and Whitehead 1993).

Cogan and others (2001) reported *S.* Enteritidis growth after 8 d at 20 °C in 7% of the whole eggs inoculated in the albumen near the shell with as few as 2 CFU. If the inoculum equaled or exceeded 25 CFU/egg when eggs were subsequently stored at 20 °C, or 250 CFU/egg when eggs were stored at 30 °C, high levels of growth of *Salmonella* in the egg occurred significantly more frequently than when the inoculum dose was smaller (Cogan and others 2001).

Chen and others (2005) compared the storage of table eggs at 4 °C, 10 °C, and 22 °C. The albumen was inoculated with 10^2, 10^4, and 10^6 *S.* Enteritidis cells. At 22 °C, for all examined concentrations of inoculum, *S.* Enteritidis was able to grow, while at 4 °C and 10 °C, its growth was inhibited, regardless of the initial concentrations used. The authors believe that storage at 4 °C and 10 °C postponed the aging process of the eggs, preserving the antimicrobial agents of the albumen, and maintaining the integrity of the vitelline membrane.

Gast and Holt (2000) determined the extent to which small numbers of *S.* Enteritidis could grow to more dangerous levels at different temperatures over a period up to 3 d. Their intention was to stimulate the potential opportunities for *S.* Enteritidis multiplication following oviposition and prior to the achievement of internal temperatures able to prevent further microbial growth in eggs. Their results showed that extensive multiplication of *S.* Enteritidis was less frequently observed at lower inoculum dose (0.1 mL containing 15 CFU of *S.* Enteritidis), shorter storage time (1 d) and lower temperatures (10 °C and 17.5 °C). At 25 °C, with higher inoculum dose (0.1 mL containing 150 CFU of *S.* Enteritidis) and longer storage time (2 and 3 d), a rapid and substantial multiplication of the foodborne pathogen occurred. The inoculation site influenced in a great extent *S.* Enteritidis multiplication, since they used 4 types of samples: yolks (internally inoculated), albumens, whole egg, inoculated at the albumen edge and whole eggs inoculated at the yolk surface. In the yolk, multiplication occurred rapidly, with *S.* Enteritidis numbers reaching 8.7 $\log_{10}$/mL, at 25 °C, after only 2 d of storage. On the contrary, it was confirmed that the albumen is not a good growth medium for bacteria, since the *S.* Enteritidis levels suffered only a slight change during storage. The whole eggs inoculated at the yolk surface presented increasing levels of *S.* Enteritidis, during storage (no matter the dose and the storage time and temperature), while the other category of whole eggs (inoculated on the albumen edge) revealed only a slight change in these levels (Gast and Holt 2000).

It is believed that *Salmonella* cells that are deposited in the albumen are able to migrate to and penetrate through the vitelline membrane, in the egg, postlay, in order to reach the yolk and thus gain access to a pool of nutrients that are necessary for its survival and growth (Baron and others 1997; Gantois and others 2009).

Braun and Fehlhaber (1995) studied the migration of *S.* Enteritidis from the albumen into the egg yolk. Different doses of *S.* Enteritidis PT 4 were inoculated on the albumen (10 to 200 bacterial cells/albumen). Storage took place at 7, 12, 20, and 30 °C for 4 wk. The results showed that *S.* Enteritidis was able to migrate from the albumen into the egg yolk during storage. The risk of egg yolk penetration was relatively low at 7 and 12 °C. However, after 14 d, at 7 °C, the 1st positive egg yolk was found. At 20 and 30 °C, the 1st positive egg yolks were already present after 1 or 2 d. Schoeni and others (1995) also observed that the temperature values of < 10 °C will not allow but a sporadic growth of *S.* Enteritidis, *S.* Heidelberg and *S.* Typhimurium in the inoculated eggs, no matter the inoculum size.

Earlier, Hammack and others (1993) showed that the growth of *S.* Enteritidis on artificially inoculated shell eggs was negligible in eggs refrigerated up to 16 d. On the contrary, the population level of this food borne pathogen increased by more than 8 $\log_{10}$ units in unrefrigerated eggs stored for the same amount of time. Lock and Board (1992) observed that when inoculating different *Salmonella* serotypes, among them *S.* Enteritidis, *S.* Typhimurium and *S.* Infantis, on egg albumen, their persistence *in vitro* was different during storage at 3 different temperatures: 4, 20, and 30 °C. The majority of serotypes remained viable but did not increase in numbers at 20 and 30 °C, for 42 d. At 4 °C, many of the serotypes died. At 20 °C, upon inoculation with 39 CFU/mL^{-1} albumen, both *S.* Enteritidis and non-*S.* Enteritidis strains were able to grow in separated fresh albumen samples up to > 10^6 CFU/mL^{-1}, during a storage period of 3 wk (Messens and others 2004; Clavijo and others 2006). It appears that the survival of *S.* Enteritidis in egg albumen is regulated by nucleic acid and aminoacid metabolism, and furthermore is related to genes involved in cell wall structural and functional integrity (Clavijo and others 2006). When extending the incubation time and increasing the storage temperature, the numbers of samples with pronounced growth increases further. Moreover, near room temperature (approximately 20 °C), the probability that an outgrowth takes place is much higher when the albumen of a fresh rather than a stored whole egg becomes contaminated with *Salmonella*. Even in the presence of a small number of *S.* Enteritidis cells present in the egg contents, cooling practices should be applied shortly after lay, to prevent *Salmonella* from growing in eggs (Messens and others 2004).

The egg yolk is a very important source of high quality nutrients, therefore fast growth of *Salmonella* is expected to occur in this site, when temperature will allow it. Experimentally infected laying hens often deposit *S.* Enteritidis on the vitelline membrane (Gast and Holt 2001; Gast and others 2007). The fast growth of *S.* Enteritidis occurs after a certain delay, during this period the integrity of the vitelline membrane being lost and finally resulting in a leakage of nutrients into the albumen. This enhances further migration and multiplication of *S.* Enteritidis in the yolk (Humphrey and Whitehead 1993). The initial growth phase potentially involves the use of iron

reserves. This appears to be sufficient to support 4 generations, but once these reserves are depleted, *Salmonella* cells would enter in a lag phase, further on translated as a stagnation in the number of bacterial cells (Gantois and others 2009). The site of deposition of *S.* Enteritidis in the shell egg could influence the extent to which this pathogen multiplies before the refrigeration would achieve growth-inhibiting internal temperature values (Gast and Holt 2001). When 10^2 CFU of *S.* Enteritidis was inoculated onto the exterior surface of intact egg yolk (the vitelline membrane), multiplication within the interior egg yolk contents occurred in 10% of the samples after 6 h of incubation, in 75% of the samples after 24 h at 25 °C (reaching mean levels of 10^4 CFU/mL) and in only 20% of the samples incubated for 72 h at 15 °C (Gast and others 2001). Further on, Gast and others (2006) tested the effect of refrigeration on the frequency of *in vitro S.* Enteritidis penetration of the egg yolk membrane. After inoculating intact exterior surface of the egg yolks with a suspension of 0.1 mL containing approximately 100 CFU, samples were held 5 min at room temperature (24 °C) to facilitate bacterial attachment to the vitelline membrane. Further on, these samples were kept at 30 °C for different periods of time (2 h, 6 h, and 24 h), followed by refrigeration at 7 °C for 18 to 22 h. *S.* Enteritidis penetrated inside the egg yolk contents in 4% of contaminated egg samples refrigerated after 2 h at 30 °C, 15% of samples refrigerated after 6 h of storage at 30 °C and 40% of samples stored at 30 °C for 24 h, followed by refrigeration. Lublin and Sela (2008) showed that from an initial concentration of 3.65 log CFU of *S.* Enteritidis inoculated into the egg yolk, the concentration increased by 1 log during the 1st 2 wk postinoculation at 6 °C, after which it remained constant, at around 4 logs, for up to 8 wk. At 25 °C, the bacterial concentrations increased to 5 logs by week 4 postinoculation and remained at 8 to 9 logs until the end.

In different European countries, cold storage of eggs is banned on the market place. The reason is related to the concept that the eggs kept in cold storage can no longer be regarded as "fresh." On the other hand, consumers are advised to keep purchased table eggs in the refrigerator until consumption (FDA 2009b). The practical aspects of this situation are different from one country to another. However, the scientific data clearly prove that refrigeration reduces in a great extent the risk of contaminated table eggs to become a vehicle for *S.* Enteritidis, a main worldwide cause of foodborne human salmonellosis.

Decontamination methods for reducing the risk of *Salmonella* spp. penetration through the eggshell and further contamination of the egg content

In the last decades, different methods have been studied for microbial decontamination of shell eggs, with a focus on *Salmonella*. We can distinguish the procedures tested for on shell decontamination from those, more limited, also active inside the shell. Moreover, concerning the 1st category, the procedures can be classified into 3 classes: the chemical, the physical and the biological procedures (Table 3).

Several procedures are currently approved by the FDA or USDA in the U.S.A. and also, commercially available for shell eggs processing facilities. As none of them is perfect, they are continuously improved, as new procedures may emerge as well. However, the need for improvement is continuous, and research should focus more on the effects on sensory, rheological, and functional properties of eggs and their acceptability by the consumers, once the decontamination was performed. Moreover, when a new method emerges, research will still be a necessity before an efficient application on a full-scale production would take place.

Table 3–Methods of shell eggs surface decontamination as postharvest control procedures for reducing the risk of salmonellosis due to *Salmonella* contaminated eggs.

Chemical methods
Washing (use of sanitizers)
Hydrogen peroxide
Electrolyzed water
Ozone
Physical methods
Irradiation
Microwave technology
Ultraviolet light technology
Pulsed light technology
Gas plasma technology
Ultrasounds
Biological methods
Plant extracts

Egg washing. In the E.U., egg washing is currently banned (with same exceptions—see further on) but this subject is always under a rigorous debate (Nys and Van Immerseel 2009). In this chapter, we will review different procedures that have the main objective of reducing or eliminating *Salmonella* spp. In order to be considered efficient, a decontamination procedure must lead to a reduction of at least 5 log CFU/eggshell^{-1}, otherwise the resulting shell eggs being regarded as inappropriate for egg safety point of view (FDA 2009b).

Egg washing is currently used in the U.S.A., Canada, Australia, and Japan, in order to reduce the bacterial contamination and to prevent the penetration of bacteria in the egg contents. Moreover, in the U.S.A., egg washing is followed by cold storage.

Washing of class A-table eggs is banned in the E.U., but still under discussion following the increase in noncage egg production. Moreover, Member States which, on June 2003 authorized packing centers to wash eggs, may continue to authorize these packing centers to wash eggs, but the eggs may only be marketed in the Member States in which such an authorization has been issued (EC 2007). For example, in Sweden, several providers are allowed to perform it, as the washing practice has been used for the last 40 y and the consumers prefer washed eggs (Hutchison and others 2003).

In the U.S.A., there are not specific guidelines provided by the FDA to the specific process of egg washing. However, FDA provides general information for the Food Service and Inspection Service (FSIS) to provide to companies and local producers, as to what types of chemicals are allowed to be used during cleaning and destaining of shell eggs. Usually, the compunds included in the list of Generally Regarded As Safe (GRAS) ones can be used without any specific limits when cleaning shell eggs. These are mentioned and described in the Code of Federal Regulations (CFR), Title 21, parts 178 to 186 under the general term of "food additives." However, for several of these so-called "food additives," limits are mentioned, and maximum allowable concentrations are described and recommended to be followed, as allowed by the food additive regulations, especially for the indirect food substances affirmed as GRAS (CFR 2012a)

In the CFR Title 7, section 56.76, there are described the minimum facility and operating requirements for shell egg grading and packing plants, point (f) clearly specifying the shell egg cleaning operations. It is stated that the temperature of the wash water shall be maintained at 90 °F (32.2 °C) or higher, and shall be at least 20 °F (6.7 °C) warmer than the internal temperature of the eggs to be washed. These values shall be maintained throughout the

entire cleaning cycle. For safety reasons, the wash water has to be changed approximately every 4 h or more often if needed, in order to maintain the sanitary conditions, and mandatory at the end of each shift. In addition, special measures have to be taken in order to avoid foaming during the egg washing operation. During the cleaning cycle, the addition of replacement water it is mandatory and has to be performed continuously. The replacement water may contain residues of chlorine or quaternary compounds, provided they are compatible with the compound used for washing. The use of iodine sanitizing water for rinse is forbidden (CFR 2012b).

Also for safety purposes, only potable water may be used during the shell eggs cleaning cycle, and it is mandatory the analysis of the iron concentration of the water supply. When the iron content exceeds 2 ppm, it has to be reduced to the maximum allowed level, and each time the water source is changed, new tests are required.

Wastewater is directly discarded, through its piping directly to the drains. Considering the type of washing procedure and equipment used, it is specified that eggs shall not be allowed to stand or soak in the water, therefore the use of immersion-type washers is forbidden. The washed eggs may be rinsed through spraying, with water having a temperature equal to, or warmer than the temperature of the wash water. It is specified that the rinse water should contain a sanitizer, approved by the national supervisor.

The main advantages of egg washing procedure are:

- the reduction of microbial load on the shell surface, minimizing the risk associated with the presence of foodborne pathogens, especially *Salmonella* spp.
- further reduction occurring after washing, since different chemicals may still be present after the washing step, continuing to exert their antibacterial effect;
- reduced risk of cross-contamination of other foods;
- reduced risk of contamination of the egg content, provided that the shell itself is not damaged.

The main disadvantage comes from the potential damage that this practice can cause to the physical barrier of the egg, especially to the cuticle (EFSA 2005). It is well known that the cuticle is the 1st defense against bacterial contamination (Board and Halls 1973).

Egg washing procedure uses water or solutions that involve chemicals (sanitizers) to determine an efficient decontamination. It is believed that different chemicals used to decontaminate the eggshell may interact with its physical barrier components. Depending on the types of chemicals used in the wash water, different microstructural changes may occur in the eggshell surfaces, and the more damaged eggshell surfaces are, the more they may allow bacterial penetration (Kim and Slavik 1996). In a study performed to investigate the abilities of different solutions, a quaternary ammonium compound (pH 7.5) and NaOCl (same pH value) succeeded in reducing bacterial penetration, without any changes to the eggshell, while Na_2CO_3 (pH 12) altered the eggshell surface which allowed bacterial recontamination (Wang and Slavik 1998). However, without using sanitizers in the washing water, it has been proven that spray washing of eggs in 15.5 °C water does not appear to increase internal shell bacterial counts (Lucore and others 1997).

Due to the concern that using a high temperature during egg washing may determine changes in egg quality, several studies have aimed to this point, evaluating as well the reduction of microbial load. Caudill and others (2010) concluded that wash water temperature did not significantly affect average Haugh Unit values, albumen height, vitelline membrane strength or aerobic bacteria in the shell matrix, but did affect average numbers of aerobic microorganisms on the exterior shell surfaces. In fact, a treatment with cool water, maintained at a pH of 10 to 12, has the potential of reducing also the internal egg temperature during and after processing, enhancing the physical qualities of the eggs, and improving their microbial quality. Using different schemes of temperature, Caudill and others (2010) obtained a reduction from 2.98 to 3.12 log CFU/mL.

Another study performed by Jones and others (2005), using 6 temperature schemes, with an exposure time of 60 s, maintaining the pH between 10.5 and 11.5, a postwashing treatment consisting of spraying a 200 ppm chlorine solution at 48.9 °C and a period of 9 wk of storage and continuous sampling, resulted in an aerobic bacterial load from 2.3 log CFU/mL to 2.87 log CFU/mL on shells and membranes, while between 53.33% and 61.8% of the samples inoculated experimentally with *S.* Enteritidis were negative. The conclusion is that washing shell eggs initially at 48.9 °C followed by a 2nd washing temperature of 23.9 °C or 15.6 °C led to a fewer aerobic bacteria present on the shell surface, than eggs washed in a combination of 23.9 °C and 15.6 °C.

Several years ago, Hutchison and others (2004) had undertaken a study on the effects of spray washing under various processing conditions to shell surface counts of *Salmonella* spp. and the presence of bacteria in egg contents. Experiments mimicked the natural conditions: they were carried out over a complete laying cycle, the eggs were contaminated with *S.* Enteritidis PT4 and *S.* Typhimurium DT104 before cuticle hardening. They used a standardized set of best washing guidelines as recommended by the equipment manufacturer and within the ranges discussed by Hutchison and others (2003). They used 2 different wash chemicals a chlorine based sanitizing agent in a concentration of 3 g/L and a quaternary ammonium based sanitizing agent in a concentration of 25mL/L, with 3 different steps in the egg washing process: prewash at 44 °C, with water flow pressure of 138 kPa; wash at 44 °C and water flow pressure of 262 kPa and rinse at 48 °C, with a water flow pressure of 262 kPa, followed by a final step consisting of air drying at 42 °C for 2 min. The used water was soft, potable and had an iron concentration of 1.4 ppb. In addition, the belt speed was 111 cm/min.

In another study that aimed to investigate the effects of different chemicals used in solution as egg disinfectants, a 1st commercial disinfecting product (pH 6.6) was used in water at 43.3 °C for 5 min, determining a 4.27 log reduction of microbial aerobic flora on eggshell. A 2nd one (pH 7.56) was used in the water at 25 °C for 10 min, determining a 3.11 log reduction. A 3rd solution of sodium hypochlorite (containing 100 ppm free chlorine, pH 8.74) used at 25 °C for 10 min, determined a log reduction of 3.08. A combination of sodium hypochlorite and 2nd solution (pH 8.4) used at 25 °C for 10 min resulted in a log reduction of 2.38. Considering side effects, the 1st compound determined a cuticle erosion, showing also an increased pore size, while in the case of the 2nd, the inside layer of the shell presented a great number of fissures and pores (Favier and others 2000).

In order to assess the food safety implications of washing table eggs under a deviation from the standard set of procedures for washing, several parameters have been modified. The results of their study have shown that when undertaken according to a strictly controlled set of best practices conditions, washing eggs that have been contaminated with *Salmonella* spp. resulted in a reduction of more than 5 log of S*almonella* spp. counts from the shell surface. In addition, this does not lead to contamination of egg contents with the foodborne pathogen.

The concentration of the washing chemical compounds, the length of the washing period, the lowered pressure of the water

flow and the age of the laying hens do not appear to influence the contamination of the egg contents. However, if the wash and rinse water temperatures are allowed to drop under 34 °C, the risk of content contamination is increased.

In commercial processing, eggs are most frequently rinsed with chlorine and chlorine-containing compounds that act as antimicrobial agents. In addition, they are widely available, have a relatively low cost and a high efficacy. Zeidler (2001a) observed that under optimal parameters, commercial egg washing can lead to a reduction of the bacterial load on the shell of 2 to 3 $\log_{10}$. A high level of chlorine can be detrimental for the quality of eggs (Bialka and others 2004) due to remaining residues deposited on the eggshell.

Hydrogen peroxide. Hydrogen peroxide (H_2O_2) is responsible for bactericidal effects in biological systems. Its toxicity is apparently due to its capacity to generate more reactive and cytotoxic oxygen species such as the radical hydroxyl (-OH), that can initiate biomolecules' oxidation. The conversion of the H_2O_2 into these toxic compounds may be potentiated by reducing agents and by paroxydases (Juven and Pierson 1996).

Padron (1995) successfully used H_2O_2 for the decontamination of hatching eggs in a challenge involving *S.* Typhimurium. The eggs were treated by double dipping in H_2O_2 at a concentration of 6%.

Cox and others (2000) reported that *S.* Typhimurium contaminated shell eggs were treated with H_2O_2 (1.4%) by immersion in a solution containing a surfactant and submitted further on to a vacuum of 12 to 13 in Hg (0.4 bar) applied for 4 min. This treatment maximized the elimination of salmonellae on fertile hatching eggs, without adversely affecting the hatchability or the early chick mortality. These results demonstrated the difficulty in killing salmonellae that had already penetrated the shell egg.

Such a treatment could be extended to table eggs, with the difference considering the immersion. This latter process should be replaced by spray washing to enhance the practicability at industrial scale.

Electrolyzed water. Water electrolysis technology was 1st used around 1900 in the soda industry, including in the production of sodium hypochlorite, being now applied in various fields and regarded as a promising nonthermal treatment for hygiene control (Al-Haq and others 2005). Electrolyzed oxidizing water (EOW) is produced by passing a diluted salt solution through an electrolytic cell, within which the anode and cathode are separated by a membrane, obtaining an acidic and an alkaline component (Huang and others 2008; Howard and others 2012). The acidic EOW may have a pH of 2 to 3, an oxidation reduction potential (ORP) of 1.150 mV and a free available chlorine concentration of up to 50 ppm, while the alkaline EOW may reach a pH of 6.8 to 11.6 and an ORP of 795 mV at the maximum value of pH (Mukhopadhyay and Ramaswamy 2012). Many studies conducted for the evaluation of the bactericidal activity of EOW have proved that it possesses antimicrobial activity on a variety of microorganisms: *Staphylococcus aureus* (Park and others 2002b), *E. coli* O157:H7 (Kim and others 2000a, 2000b), *Salmonella* Enteritidis (Venkitanarayanan and others 1999), *S.* Typhimurium and *Listeria monocytogenes* (Fabrizio and Cutter 2003), *Campylobacter jejuni* (Park and others 2002a) and others.

The antimicrobial effect of EOW is attributed mainly to pH, ORP, and HOCl (Mukhopadhyay and Ramaswamy 2012). Aerobic bacteria grow mostly at ORP range of +200 to 800 mV, while anaerobic bacteria grow well at −700 to +200 mV. The high ORP in the EOW could cause the modification of metabolic fluxes and ATP production, probably due to the change in the electron flow in cells. In general, bacteria grow in a pH range of 4 to 9. A low pH may sensitize the outer membrane of bacterial cells to the entry of HOCl, the most active of chlorine compounds. The latter appears to kill the microbial cell through inhibiting glucose oxidation by chlorine-oxidizing sulfhydryl groups of certain enzymes important in carbohydrate metabolism. Other modes of chlorine action that have been proposed are: disruption of protein synthesis; oxidative decarboxylation of amino acids to nitrites and aldehydes; reactions with nucleic acids, purines, and pyrimidines; unbalanced metabolism after the destruction of key enzymes; induction of deoxyribonucleic acid (DNA) lesions with the accompanying loss of DNA-transforming ability; inhibition of oxygen uptake and oxidative phosphorylation, coupled with leakage of some macromolecules; formation of toxic N-chlor derivatives of cytosine; and creation of chromosomal aberrations (Marriott and Gravani 2006; Huang and others 2008).

Considering shell eggs alone, a study was undertaken to compare EOW treatment with a commercial detergent-sanitizer treatment, both *in vitro*, for the decontamination of shell eggs, artificially inoculated with *S.* Enteritidis. For this *in vitro* study, eggs were soaked in alkaline EOW followed by soaking in acidic EOW at various temperatures. Treated eggs showed a reduction in population between $\geq$ 0.6 and $\geq$ 2.6 $\log_{10}$ CFU/g of shell *S.* Enteritidis. The $\log_{10}$ reduction of 1.7 for *S.* Enteritidis was observed for typical commercial detergent-sanitizer treatments, whereas $\log_{10}$ reduction of $\geq$ 2.1 for *S.* Enteritidis was achieved using the EOW treatment (Bialka and others 2004).

In a study conducted on shell eggs, performed in order to determine the effect of EOW applied using electrostatic spraying (in 4 different repetitions) on *S.* Typhimurium and other pathogenic bacterial species after applying the inoculum onto the shell eggs and allowed the bacteria to attach for 1 h, EOW completely eliminated all *S.* Typhymurium on 3, 7, 1, and 8 out of 15 eggs in 4 different treatment repetitions, respectively, even when high inoculations were used (Russell 2003).

In another study, the authors (Cao and others 2009) observed that acidic EOW is effective in reducing the populations of pathogenic microorganisms on the surface of shell eggs (aiming at *S.* Enteritidis), but its use is limited when low pH values are observed ($\leq$2.7), because dissolved Cl_2 gas can be rapidly lost due to volatilization, decreasing the bactericidal activity of the solution with time. On the other side, slightly acidic electrolyzed water (produced by electrolysis of a dilute hydrochloric acid in a chamber without a membrane), minimizes the safety issues for human health, regarding Cl_2 off-gassing. At the same time, slightly EOW reduces the corrosion of the surfaces, and because at a pH of 5.0 to 6.5, its effective form of chlorine is the HOCl, this type of EOW may result in a stronger antimicrobial activity, in comparison to acidic EOW. The same authors proved that the bactericidal efficiency of slightly acidic EOW increases with temperature, the reduction for $\log_{10}$ CFU/mL at 45 °C, after 1 min reaching less than 1.0, after an initial value of 8.0 to 8.4 $\log_{10}$ CFU/mL. After 2 min, using temperatures of 4 °C, 20 °C, and 45 °C, *S.* Enteritidis was killed (Cao and others 2009). In conclusion, the last study shows that slightly acidic oxidized water can efficiently act as a promising disinfectant agent for the shell egg washing process and the reduction or inactivation of *S.* Enteritidis inoculated on the surface of shell eggs, without environmental damages.

On the other side, Bialka and others (2004) have shown that acidic electrolyzed water did not significantly affect albumen height or eggshell strength but there were significant effects

on cuticle presence. It must be mentioned that the processing parameters of their study has much more severe effects in comparison to the slightly acidic oxidizing water processing parameters, mentioned above.

Ozone. Ozone is one of the most potent sanitizers known, active against all forms of microorganisms at relatively low concentrations (Khadre and others 2001). Due to its low stability, ozone cannot be stored, being produced on demand. At commercial level, corona discharge method is usually used. In corona discharge, 2 electrodes, one of which is the high-tension electrode and the other one the low-tension electrode (ground electrode) are separated by a ceramic dielectric medium, providing a narrow discharge gap. When the electrons have sufficient kinetic energy to dissociate the oxygen molecule, a certain fraction of these collisions occurs and a molecule of ozone is formed from each oxygen atom (Guzel-Seydim and others 2004a). Ozone destruction of bacteria is accomplished by attacking on the bacterial membrane glycoproteins and/or glycolipids, resulting in cellular components leakage and followed by cell death, through the progressive oxidation of vital cellular components, reaching the nucleic material and causing DNA-strand breaks (Guzel-Seydim and others 2004b; Perry and Yousef 2011). In addition to its bactericidal effectiveness, ozone decomposes spontaneously to O_2, hence having the advantage of being a nonpolluting sanitizer for shell eggs.

Ozone is a strong microbial agent that effectively inactivates *Salmonella* in shell eggs, its efficacy in aqueous phase being demonstrated. *Salmonella* Enteritidis was effectively inactivated $\geq$ 5 log units on the surfaces of shell eggs by high ozone concentrations (12% to 14% wt/wt O_3 in O_2 mix) (Rodriguez-Romo and others 2007). In another study involving the same serotype of *S. enterica*, ozone treatment of shell eggs, at atmospheric pressure for 3 min significantly ($P < 0.05$) reduced *S.* Enteritidis on eggshell by 3.1 log units compared with the untreated control, while longer times (up to 8 min) did not cause additional inactivation. Application of pressurized gaseous ozone for up to 20 min resulted in nonlinear inactivation of the microorganism, a trend similar to that observed when ozone was applied at atmospheric pressure. Populations of *Salmonella* Enteritidis decreased significantly ($P < 0.05$) on shell eggs treated with pressurized ozone. The 10-min treatment inactivated 4.5 and 5.9 log units or more, and the 20-min treatment inactivated 3.7 and 5.7 log units or more compared with the untreated controls (Rodriguez-Romo and Yousef 2005). On the same subject, Perry and others (2008) applied sequentially and in combination heat and ozone to shell eggs, in order to assess the log reduction of *Salmonella* Enteritidis on eggshells. *Salmonella* was recovered from all eggs treated with ozone alone and heat alone, but only 10 of 18 combination-treated eggs tested positive, indicating *Salmonella* reduction in a many of the samples. Heating shell eggs increased permeability of their membranes to ozone gas, therefore application of ozone was effective against internal *Salmonella* only when shell eggs were subjected to heat prior to ozone treatment. Also, in an attempt to differentiate the various treatments involving ozone on table eggs, Davies and Breslin (2003a) used dry and moist ozonated air, the results showing that 23 of 24 (95.8%) eggs remained contaminated after treatment compared with 11 of 12 (91.7%) controls, for the 1st one, and 4 of 12 treated eggs were contaminated compared with 9 of 12 (75.0%) control eggs for the 2nd. Therefore, the application of ozone in either type of environment was only partially effective.

Irradiation. For food irradiation, currently there are 3 types of ionizing radiation that are allowed to be used for sanitation: radiation from high-energy gamma rays, X-rays and accelerated electrons (Codex Alimentarius Commission 2003).

Gamma rays are produced by radioactive substances, called radioisotopes, among them the allowed ones being: cobalt-60 (^{60}Co) and cesium-137 (^{137}Cs). Their energy content arrives up to 1.17 to 1.33 megaelectronvolts (MeV) (^{60}Co) and 0.662 MeV (^{137}Cs). The accelerated electrons (or the electron beams) have a maximum quantum energy that does not exceed 10 MeV, being produced in linear accelerators at nearly the speed of light. X-rays, called also decelerating rays, are also produced in accelerators, their quantum energy of the electrons not exceeding 5 MeV (Riganakos 2010). The mechanism of microorganisms' inactivation is explained by the fact that ionizing rays (gamma rays) are picking electrons from the atoms of the treated product, therefore the free electrons can take part further on in the chemical reactions, also being able to destroy the DNA molecules from the living microorganisms (Riganakos 2010).

In comparison to the latter, electron beams (ionizing electrons) are more often easily accepted because there are no radioactive substances in the process (Riganakos 2010). By the acceleration to the speed if light, the electron beam gun subsequently passes the high-energy electrons onto the product, resulting in microbial activation. Electron-beam processing does not alter the temperature of the processed food and permits the application of high dose rates (10^3 to 10^5 Gy/s in comparison to only 0.01 to 1 Gy/s for gamma radiation) (Tahergorabi and others 2012). However, the depth of penetration is only 8 to 10 cm, for typical food products, therefore before irradiation of food products, the size has to be considered prior processing (Jaczynsky and Park 2003).

The X-rays are generated by interposing a metal target between the electron beam and the food product. This way, the high-energy electrons produced by the accelerator will impinge upon the metal target and produce the X-ray. The energy level is lower than in the case of electron beams, but the depth of penetration is higher (Tahergorabi and others 2012).

The scientific literature shows different attempts on shell eggs, in order to prove the efficacy of the *Salmonella* spp. inactivation.

Fresh shell eggs were inoculated with 10^8 CFU of *S.* Enteritidis with the aim of testing the effect of 3 doses of gamma-irradiation (1, 2, and 3 kGy). After the irradiation treatment, the eggs were kept at 4 °C for 42 h. The irradiation dose of 1 kGy determined a reduction of 3.9 logCFU for detectable *S.* Enteritidis on the shell. Further on, the higher used doses determined a reduction of bacterial contamination to nondetectable levels on the shell, proving the efficacy of this treatment for shell eggs surface decontamination (Tellez and others 1995).

Serrano and others (1997) tested the irradiation sensitivity of 5 *S.* Enteritidis isolates inoculated either on the surface (a level of 10^6 CFU/mL) or inside the shell eggs (by injecting 1 mL of 10^8 cells/mL). The inoculated samples were subjected to irradiation doses of 0, 0.5, 1.0, and 1.5 kGy. A minimal dose of 0.5 kGy was considered sufficient for the elimination of all the isolates from the surface. However, the same isolates showed a greater resistance when inoculated in the contents, and in this case, only the maximum dose included in the test was able to reduce *S.* Enteritidis counts by approximately 4 $\log_{10}$ in the contents.

In 2003, Wong and Kitts used low doses of electron beam irradiation (2, 3, and 4 kGy) to examine the antimicrobial effects on shell eggs inoculated with a 0.5 mL suspension of *L. monocytogenes*, *E. coli*, and *S.* Typhimurium, at a dose of 10^9 cells/mL^{-1}. After holding the inoculated samples at 20 °C for 24 h, the irradiation treatment was conducted, using the doses mentioned above. The

doses of electron beam irradiation of 3 and 4 kGy determined the reduction of the 3 pathogens to undetectable levels, with *S.* Typhimurium showing a higher resistance to irradiation, the counts decreasing slower than on the case of the other 2 species.

Using an inoculum of 10^7 to 10^8 CFU/egg, shell eggs were artificially contaminated with reference strains of *S.* Typhimurium, *S.* Enteritidis, *Campylobacter coli*, and *C. jejuni*. The range of irradiation doses for the determination of D values (the values of heat resistance for microorganisms) was 0.2 to 1 kGy for *Salmonella* spp. and 0.2 to 0.7 kGy for *Campylobacter spp.* The gamma irradiation doses were included in the range 0.5 to 5 kGy. The D values varied between 0.31 and 0.26 and 0.20 and 0.19 kGy for *S.* Typhimurium and *S.* Enteritidis, respectively, and between 0.21 and 0.18 kGy and 0.07 and 0.09 kGy for *C. coli* and *C. jejuni* for the eggshell (Cabo Verde and others 2004).

Al-Bachir and Zeinou (2006) performed another study on the irradiation of shell eggs. Using a suspension of 10^7 CFU/mL of *Salmonella* spp. the shell eggs were inoculated and subjected further on to doses of gamma irradiation from 500 to 3000 Gy, with the estimation of survival curves. The radiation dose required to reduce the *Salmonella* spp. load one log cycle (D_{10}) was 448 Gy.

Yun and others (2012) suggested another approach. They aimed to predict the optimal conditions to minimize quality deterioration while maximizing safety and functional properties of irradiated eggs, by combining different concentrations of chitosan coatings and different ionizing radiation doses. In a 1st step, eggs were coated with chitosan, using concentrations of: 0.0%, 0.5%, 1.0%, 1.5%, and 2.0%. The 2nd step consisted in the inoculation of the shell eggs, through dipping, with *S.* Typhimurium and further on subjected to an irradiation treatment, using doses of: 0.0, 0.5, 1.0, 1.5, and 2.0 kGy. The results showed that using doses of more than 0.5 kGy, in combination with concentrations of more than 1% chitosan, *S.* Typhimurium was successfully removed from the eggshell. Moreover, foam stability, foaming capacity and Haugh units are not negatively affected when using a 0.45 kGy irradiation dose and a concentration of 0.525% chitosan coating.

Microwave technology. Microwaves are oscillating electromagnetic waves with frequencies in the 300 MHz to 300 GHz range.

The effects of microwaves on pathogens can be generally expressed in 2 forms: thermal and nonthermal. Thermal inactivation is caused by heating during the microwave application process, involving changes such as denaturation of enzymes, proteins, nucleic acids or other vital components as well as disruption of membranes. Nonthermal effects have been classified in 4 categories:

(a) selective heating, explained by the fact that microwaves heat solid microorganisms more effectively than by the surrounding medium, causing a more rapid killing of the organism;

(b) electroporation, caused when an electrical potential crosses the membrane of the microorganism, determining the formation of pores in the membrane, and a further leakage of cellular components;

(c) cell membrane rupture, due to the voltage drop across a membrane;

(d) magnetic field coupling, caused by a disruption in internal components of the cell, leading further on to cell lysis (Datta and Davidson 2000; Leonelli and Mason 2010).

Microwaves can be used to reduce the load of different bacteria found on the eggshell, among them *S.* Enteritidis, as Lakins and others (2008) already showed. Using a new directional microwave technology (ITACA New Tech, Brescia, Italy), the eggs were exposed to 2.45 GHz, corresponding to 12.2 cm wavelength, for 20 s. A CO_2 treatment for 30 s was performed at the end of the microwave processing. The maximum reduction of *S.* Enteritidis on the eggshell was of approximately 2 log cycles, this value being considered by the authors as appropriate to eliminate *S.* Enteritidis in most naturally contaminated eggs. However, further studies are required to reach a minimum reduction of 3 to 4 $\log_{10}$.

Ultraviolet light technology. Ultraviolet (UV) light occupies a wide band of wavelengths in the nonionizing region of the electromagnetic spectrum between X-rays (200 nm) and visible light (400 nm), but only UV in the range of 250 to 260 nm (short-wave UV radiation, or UVC) may be lethal to most microorganisms. Among its practical applications may be mentioned: inhibition of microorganisms on surfaces, destruction of microorganisms in the air and sterilization of liquids (Bintsis and others 2000). UV radiation inactivates microorganisms by inducing a cross-linking between pyrimidine nucleotide bases in the DNA, this resulting in inhibition of DNA transcription and replication mechanisms, leading eventually to microbial cell death. In addition, it has been demonstrated that UV radiation affects cell membrane integrity, inducing protein modifications and inhibiting oxidative phosphorylation (Rodriguez-Romo and Yousef 2005).

Using UV pulsed light (3 times per second, each pulse's duration 360 μs) of 3800 V input voltage, Keklik and others (2009) generated 1.27 J/cm^2/pulse of radiant energy at 1.5 cm below the lamp surface. Samples consisting of shell-eggs artificially contaminated with *S.* Enteritidis were subjected to different treatment periods and different distances were also used (1, 3, 5, 10, 15, 20, and 30 s at 9.5 and 14.5 cm). Results showed that at a treatment distance of 9.5 cm from the UV-strobe, the reduction was between 2.0 and 5.3 CFU/cm^2 and the visual appearance of samples did not show any difference after treatments. Treatments for 3, 5, and 10 s were not significantly different ($P < 0.05$), while 10 s treatment was not significantly different from 15 s treatment ($P > 0.05$). The results for 20 s and 30 s were significantly different from other treatments ($P < 0.05$), and considering the distances, the treatments at 9.5 and 14.5 cm were not significantly different ($P > 0.05$) regardless of the treatment times. The treatment with the shortest time that resulted in negative enrichments was the one comprising the distance of 9.5 cm.

Treatment of *Salmonella*-contaminated shell eggs with UV radiation (100 μW/cm^2) for 2 and 4 min significantly ($P < 0.05$) decreased *S.* Enteritidis population by 2.6 and 2.0 log units, respectively, compared with the untreated controls. In the same study, but another trial, *Salmonella*-contaminated shell eggs were treated with higher UV radiation intensity (1500 to 2500 μW/cm^2) for up to 5 min; this treatment resulted in significant ($P < 0.05$) microbial reductions; UV treatments for 1, 3, and 5 min decreased *Salmonella* populations by 3.4, 3.0, and 4.3 log units, respectively, compared with the untreated controls; no significant difference ($P > 0.05$) was observed when reductions in *Salmonella* populations after 1, 3, and 5 min of irradiation were compared (Rodriguez-Romo and Yousef 2005).

Using a hand-operated egg roller, an UV treatment consisting of 254 nm light, at 7.35 mW/cm^2, for 0, 15, 30 and 60 s, was applied to shell eggs, finally assessing APC, in order to observe the reduction of microbial load on the eggshells. In all 30-s UV exposure trials, there was a significant reduction of 1 to 2 $\log_{10}$ CFU/egg, compared to the controls. Eggs rotated for 60 s had significantly greater reductions of APC than the other time intervals of exposure (a 2 to 3 $\log_{10}$ CFU/egg of aerobic microorganism

compared to controls was observed after 60 s of exposure to UV radiation) (Chavez and others 2002).

Using an UVC (254 nm) dose rate of 10 mW/cm^2/s at a 20 cm distance from the bulbs and irradiation by 90° rotation 4 times during exposure, Sommers and others (2010) obtained different log reduction per J/cm^2 of *Salmonella* spp. on shell eggs: 0.43 ± 0.21 at 0.5 J/cm^2; 0.31 ± 0.2 at 1 J/cm^2; 0.53 ± 0.52 at 2 J/cm^2 and 0.98 ± 0.55 at 4 J/cm^2.

Pulsed light technology. Pulsed light (PL) treatment is a nonthermal technology that consists of the application of short duration pulses of an intense broad spectrum light (200 to 1000 nm). This part of the spectrum is mainly responsible for the lethal effect of the PL, through photochemical and/or photothermal mechanisms. The photochemical damage produced on the bacteria is induced mainly on DNA, by the UV-C region of the spectrum (200 to 290 nm), while photothermal damage is due by the absorption of light by microorganisms, which causes a temporary overheating leading to the vaporization of water inside the cell and the rupture of the membrane (Wekhof and others 2001; Woodling and Moraru 2005). Hierro and others (2009) showed that the inactivation of *S.* Enteritidis by using PL delivered in 100 μs, with 30% of the spectral output corresponding to UV light, is possible. For this, they used washed and unwashed eggs, in order to observe also the effect of the absence/presence of the cuticle. Dipping unwashed eggs into the culture provided an initial contamination of 4.5 log units in the shell. For this category, the PL treatment determined a reduction of 3.6 logCFU/egg in 24% to 80% of the eggs. For washed eggs, the inoculation determined an initial contamination of 6.3 log units, while the maximum reduction obtained was of only 1.8 logCFU/ egg. This method does not pose any risk for the egg quality, as the maximum temperature increase recorded in the eggs was 3 °C when 12 J/cm^2 were applied. The lower contamination obtained in washed eggs supports the hypothesis that the state of the cuticle influences the utility of the treatment. Therefore, any circumstance that causes the loss of integrity of the cuticle reduces the efficacy of PL treatment.

Using also unwashed eggs, by inoculation with *S.* Enteritidis and treatment with 8 flashes of 0.5 J/cm^2, an 8 log reduction was observed on the surface of the shell eggs. The same author observed than when using an inoculum solution colder than the egg, a deeper penetration of the microorganisms into the shell was enhanced, while the inactivation yielded 2 to 4 folds lower log reductions, in comparison to the 1st experiment (Dunn 1996).

Gas plasma technology. Plasma is constituted by particles in permanent interaction: photons, electrons, positive and negative ions, atoms, free radicals and excited and nonexcited molecules. Based on the conditions in which they were created, plasma can be thermal and nonthermal. Thermal plasmas are obtained at high pressure and need substantial power to be conserved, while nonthermal ones are obtained at lower pressure, use less power and are characterized by an electron temperature which is much higher than that of gas (Moisan and others 2001; Moreau and others 2008).

During plasma treatment, microorganisms are exposed to an intense bombardment by the radicals of OH and NO, but the mechanism of their inactivation is not entirely known. The treatment probably provokes surface lesions that the living bacterial cell cannot repair sufficiently quickly. The process involved in microorganism destruction can also be represented by the absorption of the plasma components onto the surface of microorganisms, forming volatile compounds that are then eliminated from the cells. Also, plasma induces perforations in the membranes of microorganisms and provokes a marked acidification of the medium (Laroussi and others 2003; Laroussi and Leipold 2004).

Gas plasma can represent a good opportunity for the decontamination of foods as an alternative method for those products that cannot be sanitized by conventional methods. In the European Union, washing or cleaning of shell eggs before or after grading is banned; therefore the need for alternative methods is rising. Ragni and others (2010) studied the possibility of using a nonthermal gas plasma device to decontaminate the surface of shell eggs. The device was represented by a resistive barrier discharge system, which comprises 2 electrodes. One or both of them are covered by a high resistive material, which would prevent arcing. The efficacy of the prototype for superficial decontamination was evaluated by exposing shell eggs artificially inoculated with *S.* Enteritidis and *S.* Typhimurium to gas plasma for different times: 0, 10, 20, 30, 45, 60, and 90 min. For *S.* Enteritidis, an exposure of 10 to 20 min resulted in a decrease of 1.0 to 1.6 log CFU/eggshell, in comparison to untreated samples. A maximum reduction of 2.2 to 2.5 log CFU/eggshell were observed following 60 to 90 min, at a relative humidity (RH) of 35%, while at RH 65%, the effectiveness of the treatments was enhanced. The efficacy of the gas plasma generator increased by increasing the treatment time, this showing a quasi-linear trend. For *S.* Typhimurium, a higher sensitivity was observed when using 65% RH. Also, a significant reduction of 3.5 log CFU/eggshell was observed when treated for 90 min.

Kayes and others (2007) studied the efficacy of another gas plasma generator device using one atmosphere uniform glow discharge for inactivation of foodborne pathogens, showed that the microbial load of different bacterial species (*E. coli* O157:H7*, L. monocytogenes, Staphylococcus aureus, B. cereus, S.* Enteritidis*, Vibrio parahaemolyticus, Yersinia enterocolitica, Shigella flexneri*) was strongly reduced during an initial exposure time of 30 to 90 s. However, no appreciable differences between Gram-positive and Gram-negative pathogens were observed, although the spore-forming *B. cereus* was more resistant to plasma than the non-spore-forming species (Kayes and others 2007).

Ultrasounds. Ultrasound treatment of food products is a useful tool to minimal processing, due to the fact that the transfer of acoustic energy is instantaneous and distributed throughout the whole volume of the products (Ulusoy and others 2007). The mechanism of microbial killing by ultrasonic waves is mainly due to the thinning of cell membranes, localized heating and production of free radicals (Piyasena and others 2003). Micro-mechanical shock waves are created by making and breaking microscopic bubbles induced by fluctuating pressures under the ultrasonication process; these shock waves disrupt cellular structural and functional components and lead to cell lysis (Ulusoy and others 2007). The sonication process determines microbial destruction as it follows: by creating regions of alternating compression and expansion, the longitudinal waves cause cavitation to occur, and bubbles are formed; by expansion, they reach a point where the ultrasonic energy provided is not sufficient to retain a vapour phase, and therefore, rapid condensation occurs. The condensed molecules collide violently, creating shock waves; these waves create regions of very high temperature and pressure, reaching up to 5500 °C and 50 MPa. Different combinations of this technology with other treatments have been proposed: thermosonic (heat plus sonication), manosonic (pressure plus sonication), and manothermosonic (heat plus pressure plus sonication), all of them representing highly efficient methods to inactivate microbes, as they are more energy-efficient and effective in killing microorganisms

(Dolatowski and others 2007). Ultrasonic method was applied efficiently on *Salmonella* Enteritidis, by shell eggs treatment, in combination with thermal treatment. The parameters used were: 54 °C for 5 min, 24 kHz and 400 W at 60 μm. *S.* Enteritidis count was reduced ($P < 0.05$), from 7.78 log CFU/eggshell to 2.95 log CFU/eggshell. There was a negligible effect of thermoultrasonic treatment on the eggshell morphology and structures, while the cuticle suffered some changes in its morphology, but without effect on storage conditions and bacterial growth detected in the content of eggs (Cabeza and others 2011).

The use of plant extracts. The consumers' demand for organic and nonprocessed food products is increasing; therefore the use of plant extracts for table eggs decontamination may be considered a suitable option, from this point of view.

Recently Krittika and Gi-Hyong (2012) have published a review on the inhibitory effects of several plant extracts on *Salmonella* spp. According to these authors, the phenolic compounds are responsible for their bactericidal effects as they interact by permeabilizing the membrane. Their biological activity seems to depend also on the solvent used for extraction.

Currently, very few studies provided published results on this subject, especially on shell eggs. Davies and Breslin (2003a) mentioned a natural herb extract that has an inhibitory effect on *Salmonella* and other harmful bacteria. When eggs previously contaminated with *Salmonella* Enteritidis were dipped in a 2% Protecta II (Bavaria Corp. Intl., Apopka, Fla., U.S.A.) and further on air-dried at room temperature, the authors did not observe a difference in the number of eggs that remained contaminated (8/20), compared with the distilled water control (8/20).

Recently, Pohuang and others (2009) have tested the effect of an ethanolic extract of *Punica granatum* L. against *Salmonella* Enteritidis on eggshells and eggshell membranes. Using a concentration of 1.25% and one of 2.5% (w/v) of this alcoholic plant extract applied for 10 min did not lead to a complete elimination of *S.* Enteritidis on both eggshells and eggshell membranes.

The effectiveness of these plant extracts has not been fully demonstrated until now.

Conclusions

The use of different preventive methods has the effect of reducing the likelihood that eggs become contaminated with *Salmonella* spp., especially with *S.* Enteritidis. On the farm level, the different preharvest methods may reduce the risk of egg contamination by interfering in the infection process and reducing the likelihood of this foodborne pathogen penetration in the forming egg. Further on, postharvest methods may reduce the risk of human salmonellosis, by respecting the refrigeration step and by different procedures, either chemical or physical. These latter reduce the existing bacterial counts, especially on the eggshell and ensure the microbiological quality of the shell eggs marketed in different parts of the world. However, these postharvest chemical or physical procedures are not worldwide accepted and implemented, as research is still needed on this topic, to ensure that the nutritional quality and properties of shell eggs are maintained, no matter the processing methods applied.

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