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Identifying risk factors for eggshell contamination by *Bacillus cereus* group bacteria in French laying farms

A.Z. KONE, S. JAN, C. LE MARECHAL*, N. GROSSET, M. GAUTIER, J. PUTERFLAM¹ AND F. BARON¹

Equipe de microbiologie de l'œuf et des ovoproduits, Agrocampus Ouest, UMR1253 Science et Technologie du Lait et de l'Œuf, F-35042 Rennes, France, and ¹ITAVI, 22400 Ploufragan, France

Abstract 1. The growth of *Bacillus cereus* group bacteria often limits the shelf-life of pasteurised liquid egg products and is also a putative toxin producer. This study was performed to better understand the route of contamination by *B. cereus* in egg products by studying the factors affecting eggshell contamination on-farm. 2. Eggs were collected in warm and cold seasons in 50 conventional laying farms in Western France. Egg surfaces were analysed for the presence of *B. cereus* group bacteria, environmental measurements were recorded and production practices were identified through a questionnaire filled out by the farmers. 3. A total of 44% of the farms were contaminated by mesophilic and 10% by psychrotrophic *B. cereus* group bacteria. No significant effect of the season was observed, whatever the thermal type. Several procedures were associated with reduced eggshell contamination by mesophilic bacteria, including the limitation of dust formation from manure and feeding and efficient disinfection of the silo, houses and the sanitary wall between houses. 4. The research highlights the need to promote prevention strategies, from farm to fork, for the control of eggshell and putative subsequent egg product contamination by *B. cereus* group bacteria.

INTRODUCTION

Approximately 25% of French egg production is transformed into liquid egg products, which are used in highly diversified sectors for their functional properties. The microbial quality of shell eggs and egg products is of particular importance when they are used for the manufacturing of susceptible food such as cream, dessert, dairy, meat and seafood products. The contamination by *Salmonella*, while remaining the major safety concern of producers, is now controlled by surveillance at the farm level and under egg product pasteurisation at the industrial level. However, eggs and egg products remain the major source of confirmed food poisoning in France (Delmas *et al.*, 2006). The heat treatments usually applied for egg product pasteurisation are moderate because of the heat sensitivity of the egg proteins, and they

are also inefficient in controlling spore-forming bacteria. Among spore formers, bacteria belonging to the *Bacillus cereus* group frequently contaminate the raw materials used for food production, because of their wide distribution in nature (see Anderson *et al.*, 1995, for review). These types of microorganisms are well-known in the food industry, and particularly as food spoilers in the dairy industry, because of their various enzymatic activities, leading to heavy economic losses. Moreover, several strains of the group are known to synthesise toxins responsible for food-borne diseases or are able to multiply at low temperatures. Although the processes may resemble those of dairy product manufacturing, for which the literature is abundant, there are relatively few studies referring to the egg product processing sector (Baron *et al.*, 2007; Pina-Pérez *et al.*, 2009; Jan *et al.*, 2011). The contamination by *B. cereus* group bacteria is of

Correspondence to: Dr Florence Baron, Equipe de microbiologie de l'œuf et des ovoproduits, Agrocampus Ouest, UMR1253 Science et Technologie du Lait et de l'Œuf, 65 rue de Saint-Brieuc, 35042 Rennes, France. E-mail: florence.baron@agrocampus-ouest.fr

*Present address: ANSES, Laboratoire de Ploufragan-Plouzané, Unité Hygiène et Qualité des Produits Avicoles et Porcins, Ploufragan, France.

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particular concern for this sector because storage of pasteurised liquid egg products at refrigeration temperatures, for shelf-lives ranging from a few weeks to three months, may lead to spoilage. In addition, the question of health issues cannot be neglected since a strain of this group, isolated from a spoiled liquid egg product, has already been shown to produce toxins (Baron *et al.*, 2007).

Risk factors, including production practices and season, were investigated for the contamination of the raw materials (i.e. the surface of shell eggs) by psychrotrophic and mesophilic bacteria of the *B. cereus* group. Eggs were collected in both warm and cold seasons in 50 conventional laying farms in Western France, in parallel with measurements of ambient temperature, relative humidity, dust concentration and investigation of production practices. The presence of both mesophilic and psychrotrophic bacteria was assessed after enrichment of the eggshell flora at 37°C and 10°C, respectively.

MATERIALS AND METHODS

Egg sampling and data collection in the laying farms

Egg sampling

Fifty laying farms belonging to the major group of egg producers in Western France (36% of the national production in 2003) were visited from May 2007 to February 2008. In each farm, 60 eggs were randomly collected from each house. This size of sample was shown to provide statistically relevant results by De Reu *et al.* (2005). The eggs were collected on disinfected plastic trays placed in disinfected boxes and transported at ambient temperature to the laboratory for microbial analysis.

Environmental measurements

In parallel with egg collection, temperature and hygrometry were measured inside the house. The dust concentration was measured in the centre of the house with a vacuum pump (Prolabo, Fontenay sur Bois, France) linked to a filter (47 µm) and a volumetric counter. The dust concentration was expressed as follows: (mass of the filter at the end of egg collection – initial mass of the filter) m³ of drawn air during the time of the visit.

Investigation of production practices

A questionnaire containing about 50 items was submitted to each farmer to provide additional information about the flock (Table 1). The resulting data set contained information on the sampling season (cold or warm), several housing characteristics (housing systems, age, number and types of buildings and presence of other productions in the farm), practices of production

Table 1. Description of the layer house characteristics and the production and hygiene practices in the 50 visited farms in Western France

Characteristic	Modality	Percentage
Number of houses	1	60
	2	32
	3	8
Other animal productions	Pork production	74
	Other avian productions	21
	Cow production	5
Age of the houses (years)	25	56
	26–40	44
Type of cages	Conventional	44
	Furnished	56
Number of cages per house	<4000	30
	4000–8000	32
	>8000	28
	Unanswered	10
Number of hens per cage	<6	38
	6–12	40
	12–18	12
	>18	10
Number of floors per cage	<5	58
	>5	40
	Unanswered	2
Hen strain	Isa Brown	76
	Lohmann	20
	Shaver	2
	Hi-line	2
Origin of the pullets	Same flock	48
	Different flocks	42
	Unanswered	10
Age of the hens at the time of visit (weeks)	<20	2
	20–30	20
	30–40	14
	40–50	18
	50–60	24
	>60	20
Mortality (%)	Unanswered	2
	<0.5	18
	0.5–1	12
	1–1.5	14
	1.5–2	12
	2–2.5	8
Food presentation	>2.5	20
	Unanswered	16
	Flour	52
System of feeding	Crumbs	48
	Carriage	30
Preventive treatment	Chain plate	70
	Yes	40
Number of persons working in the house	No	60
	1	5
	2	29
	3	14
Farmer working in another building	>3	2
	Yes	58
Hygiene of the sanitary wall	No	42
	Good	50
Farmer hygiene	Bad	50
	Good	74
Frequency of manure evacuation	Bad	26
	Once a day	62
Sanitation duration	Once a week	38
	<3 weeks	34
Cleaning	3 weeks	32
	>3 weeks	30
	By the farmer	82

(Continued)

Table 1. (Continued)

Characteristic	Modality	Percentage
Phase of wet cleaning	By an independent company	18
	No	82
Disinfection	Yes	18
	By the farmer	30
Type of disinfection of the house	By an independent company	70
	Pulverisation (spray treatment)	48
	Thermonebulisation (steam treatment)	52

(origin, age and feeding of the laying hens, prophylaxis), practices of hygiene (manure evacuation, cross-contamination, sanitary walls, practices of sanitation and disinfection).

Microbial analysis

Enrichments

One to 5 d after collection, the eggs were pooled into batches of 10 eggs to form 6 batches per farm. For each batch, eggs were broken and eggshells were crushed with a pestle, and 10 g of crushed eggshells were transferred into 90 ml peptone water (AES, Combourg, France) containing 5 g/l lithium chloride (Prolabo, Saint Herblain, France) and homogenised with a Stomacher (AES). This procedure was repeated twice. The first enrichment medium was incubated at 37°C for 24 h to favour the growth of mesophilic bacteria and the second one was placed at 10°C for 5 d to favour the growth of psychrotrophic bacteria. At both temperatures, each enrichment coming from one batch of 10 eggs was considered a sample. The results were expressed as the percentage of positive samples over all samples (6 samples × 29 farms at the warm season and 6 samples × 21 farms at the cold season) at each enrichment temperature and also as the percentage of positive farms at each enrichment temperature.

DNA extraction

A sample of 5 ml of each enrichment was transferred into an Eppendorf tube containing 300 µl of lysis buffer, corresponding to a 25% solution of chelex beads (Sigma, St Quentin Fallavier, France) in molecular biology quality water (Sigma). After centrifugation at 4000 g for 7 min at 4°C, the pellet was washed and re-suspended in 200 µl of molecular biology quality water (Sigma). Bacterial lysis was carried out by heating the solution at 100°C for 10 min. The solution was then centrifuged at 4000 g for 7 min at 4°C. The supernatant was transferred into a new Eppendorf tube and

centrifuged under the same conditions to purify the DNA from the remaining cell debris. The solution was diluted tenfold before real-time PCR assay.

Real-time PCR

After DNA extraction, the presence of an *sspE* gene sequence, specific of *B. cereus* group bacteria, was assessed by real-time PCR, using the *sspE1-F* and *sspE1-R* primers (5'-GAAAAAGATGAGTAAA AAACAACAA-3' and CATTGTGCTTTGAATGC TAG-3', respectively), as described by Kim *et al.* (2005). The I5 *B. cereus* strain, generously given by Dr M.H. Guinebrière (INRA, Avignon, France), was used as the positive control after DNA extraction. Reactions were carried out in duplicate in a mix containing 5 µl of the DNA solution (200 ng/µl), 10 µl of 2× AbGene buffer (Sigma, Saint Quentin Fallavier, France), 3 µl of molecular biology quality water (Sigma) and 1 µl of each primer (Sigma) at a concentration of 10 µM. The amplification reactions were carried out in a Biorad-iCycler (Biorad, Marnes La Coquette, France) as follows: 15 min at 95°C followed by 45 cycles of 15 s at 95°C, 2 s at 58°C and 25 s at 72°C, as described by Kim *et al.* (2005). Results were expressed as presence (positive sample) or absence (negative sample) of mesophilic (enrichments at 37°C) and psychrotrophic (enrichments at 10°C) *B. cereus* group cells.

Statistical analyses

The data obtained from the questionnaire and by the environmental measures were considered as qualitative values, and their co-variance with the active nominal reference data (presence/absence of *B. cereus* group bacteria after enrichment at 10°C or 37°C) was tested by the homogeneity Pearson chi-square test of independence ($P < 0.05$, Statview). Before this, co-variance between the variables had been tested for the exclusion of redundant variables or for their prioritisation. The sole variables presented in the results section are the variables that are not redundant.

RESULTS AND DISCUSSION

Description of the egg-producing poultry farms

Of the 50 items of the questionnaire, only the major factors were chosen for presentation (Table 1). The egg-producing poultry farmers answered at a level of at least 90%, except for the question concerning hen mortality (response rate 84%). Concerning housing characteristics, the results presented in Table 1 showed that the farms had mainly a single house (60%) or two

houses (32%) and 38% of the farms included other animal productions, mainly pork production (74%). The layer houses were at least 25 years old. Hens were housed in conventional laying cages (44%) and in furnished cages (56%), which was representative of the French layer housing described by Huneau-Salaün *et al.* (2008) and corresponded to the evolution from conventional to furnished laying cages, as prescribed by the EU 1999/74/EC Directive.

The number of cages per house, the number of hens and floors per cage were separated into different classes (Table 1). The minimum number of cages per house was 830 and the maximum was 14 440. The minimum number of hens by cage was 4 and the maximum was 60. The number of floors per cage was less than 5 for 58% and more than 5 for 40% of the houses.

The farms mainly housed ISA Brown (76%) and Lohmann (20%) strains, and few Shaver (2%) and Hi-Line (2%) strains were represented. For 48% of the farms, the pullets came from the same flock and for 42%, from different flocks. The age of the hens were also classified (Table 1) and were between 18 and 73 weeks old at the time of the visit.

The level of mortality varied depending on the farm (Table 1), but was always less than 2.5%. This was better than the 7.2% and 5% described in the studies of Moinard and Morisse (1997) and Koehl and Guin (1996), respectively. This low level of mortality could be ascribed to the absence of health disorders in the farms, but it is important to note that this question was answered by only 84% of the farmers. These results should, therefore, be considered with caution.

The food, presented in the form of meal (52%) or crumbs (mash) (48%), was distributed several times a day by using a carriage (30%) or a chain (70%). Water was given *ad libitum*. Preventive treatments and additives (vitamins A, D₃ and E; phytase, glucanase and xylanase; worming) were administered in 40% of the farms.

Considering the hygiene practices in the farms, 1–7 persons worked in the houses with a majority of 2 persons (29%). In 58% of the farms, the same farmer worked in different buildings. All the farms were equipped with a sanitary wall correctly used on 50% of the farms. A total of 74% of the farmers changed clothes and washed their hands before entering the house.

Dry manure was evacuated daily for 62% and stored outside of the building for 96% of the farms in accordance with the principles of good hygiene by separating manure from animals (Drouin, 2000).

Sanitation practice varied from one farm to another: 34% of the farmers practising sanitation less than once in 3 weeks, 32% once in 3 weeks and

30% more than once in 3 weeks. These results were similar to those obtained by Huneau-Salaün *et al.* (2008), who noted an average of 20 d separating two sanitation procedures: this period maintained clean and disinfected pipes and equipment.

The cleaning was carried out by the farmer himself (82%) or by an independent company (18%). The cleaning phase usually resulted in a dry cleaning for dust removal and was complemented by a wet cleaning for 18%. The disinfection was mainly carried out by an independent company (70%) or by the farmer himself (30%). The disinfectant was applied by a steam treatment, that is thermonebulisation (52%), or by a spray treatment, that is pulverisation (48%).

Effect of the season on the occurrence of mesophilic and psychrotrophic *B. cereus* group bacteria on eggshell surface

Table 2 presents the results of the detection of mesophilic (enrichments at 37°C) and psychrotrophic (enrichments at 10°C) *B. cereus* group bacteria on the surface of 174 samples. Whatever the thermal type, no significant effect of season was observed. The level of contamination was higher for the enrichments realised at 37°C than at 10°C, highlighting a higher occurrence of mesophilic bacteria in the samples. While around half of the laying farms was contaminated by mesophilic bacteria, with an average of 13.0% positive samples, only 10.0% of the farms was contaminated by psychrotrophic bacteria, with only 2.3% positive samples.

In the field of egg production, the type of eggshell contaminants was shown to vary quantitatively and qualitatively depending on the geographical area (Mayes and Takeballi, 1983) and on the season (De Reu *et al.*, 2005; Wales *et al.*, 2007), with a lower extent during the winter period. The total mesophilic aerobic flora, mainly estimated by classical microbiological analyses, has been examined in the literature, particularly food-borne pathogenic bacteria such as *Salmonella*. Depending on the study, the level of eggshell contamination ranged from 10^{3.8} to 10^{6.3} CFU mesophilic aerobic bacteria per egg (Moats, 1980; Lucore *et al.*, 1997;

Table 2. Percentage of positive samples and positive farms at warm and cold seasons for the detection of *B. cereus* group bacteria after eggshell enrichment at 37°C (mesophilic bacteria) and 10°C (psychrotrophic bacteria)

Season	Enrichment at 37°C		Enrichment at 10°C	
	Positive samples (%)	Positive farms (%)	Positive samples (%)	Positive farms (%)
Warm	14.9	48.3	1.7	6.9
Cold	10.3	38.1	3.2	14.3
Total	13.0	44.0	2.3	10.0

Favier *et al.*, 2000; Jones *et al.*, 2004; De Reu *et al.*, 2005; Musgrove *et al.*, 2005; De Reu *et al.*, 2006). Mayes and Takeballi (1983); Board and Tranter (1995) and Protais *et al.* (2003) described Gram positive bacteria as the major flora of eggshells. Moats (1980) isolated *Streptococcus*, *Aerococcus*, *Micrococcus*, *Staphylococcus* and *E. coli* by conventional bacteriological methods. De Reu *et al.* (2006) observed a predominance of *E. coli* and *Staphylococcus* by 16S DNA sequencing.

We note that none of these studies have identified eggshell contamination by the *B. cereus* group. This study, therefore, represents the first investigation of these Gram positive spore-forming bacteria in the area of egg production. Several studies have investigated the prevalence of *B. cereus* group bacteria in raw milk and in dairy cow houses (Magnusson *et al.*, 2007a, 2007b; Vissers *et al.*, 2007; Bartoszewicz *et al.*, 2008). According to these authors, the concentration of *B. cereus* spores was relatively low in raw milk. Te Giffel *et al.* (1997) and Bartoszewicz *et al.* (2008) also showed a low concentration of spores in milk after manufacturing and pasteurisation (5 CFU/ml and 0.1 CFU/ml, respectively). However, Te Giffel *et al.* (1997) isolated *B. cereus* group cells in 40% of pasteurised milk samples obtained from household refrigerators, and the counts were less than 5 CFU/ml in the positive samples. Larsen and Jorgensen (1997) observed a large contamination of bottles of pasteurised milk kept 8 d at 7°C (56% positive bottles). In the positive bottles, the counts varied from 10 to 3.1⁵ CFU/ml.

For both milk samples and samples coming from dairy cow houses, a higher level of contamination was described at the warm than at the cold season, probably due to a higher risk of contamination of the cow teats through the soil in the grazing period, according to Christiansson *et al.*

(1989). Von Stetten *et al.* (1999) found a higher concentration of psychrotrophic *B. cereus* group bacteria in alpine than in temperate and tropical soils, suggesting a selective effect of the temperature. In our study, hens were housed throughout the year in houses at controlled temperatures, and this is probably the reason why we did not observe any effect of the season and also why we observed their maintenance as the dominating thermal type during the cold season. Even if their occurrence was twofold higher at the cold season, the number of positive samples of psychrotrophic bacteria was too low to assess the statistical significance of this seasonal effect.

The results showed a generally low frequency of eggshell contamination by *B. cereus* group bacteria. This could be ascribed to the fact that the eggs were collected just after laying. Hence, this contamination was probably not representative of the contamination of the eggshell surfaces just before egg breaking in the industry.

Identification of the risk factors for eggshell contamination by *B. cereus* group bacteria

The percentage of farms contaminated by psychrotrophic *B. cereus* group bacteria was too low (Table 2) to carry out a statistical analysis, and risk factors were analysed exclusively for contamination by mesophilic bacteria (Table 3).

The high frequency of dry manure evacuation ($P < 0.05$, Table 3) was the main risk factor for contamination of the eggshell surface, with 61.3% positive results for the farms removing the manure once a day compared with 15.8% for the farms removing the manure once a week. Dry manure evacuation may be responsible for dust dispersion, including *B. cereus* group spores that may deposit on the eggshells. The results also

Table 3. Risk factors for eggshell contamination by mesophilic *B. cereus* group bacteria determined with the Pearson chi-square test of independence (df= 1)

Variable	Modality	Positive farms for <i>B. cereus</i> (%)	P value (χ^2)
Frequency of manure evacuation	Once a day	61.3	0.002
	Once a week	15.8	
Farmer working in another building	Yes	55.2	0.023
	No	22.2	
Draining of the feeders	Yes	21.4	0.040
	No	52.8	
Pullets from the same flock	Yes	58.3	0.045
	No	28.6	
Silo disinfection	Yes	35.3	0.07
	No	62.5	
Type of disinfection of the house	Pulverisation ^a	56.5	0.1
	Thermonebulisation ^b	37.5	
Dust concentration in the house	≥0.02 mg/m ³	60.0	0.1
	<0.02 mg/m ³	36.0	
Hygiene of the sanitary wall	Good	35.3	0.1
	Bad	60.0	

^aSpray treatment. ^bSteam treatment.

showed that 60% of the farms had dust concentrations greater or equal to 0.02 mg/m³ and only 36% had lower dust concentrations (Table 3). There was a trend ($P = 0.1$) for a direct effect of the dust concentration, and it was concluded that a direct relationship between the frequency of dry manure manipulation and dust formation inside the houses exists. This is consistent with the suggestion of Mollenhorst *et al.* (2005), who showed that manure desiccation, by increasing dust dispersion, slightly enhanced the risk of flock infection by *Salmonella enteritidis* for hens maintained in cages.

The absence of feed removal represented a significant risk of contamination ($P < 0.05$, Table 3), with 61.3% positive results for the farms removing old feed as opposed to the 21.4% for farms distributing fresh feed without removal of feed. Similarly, there was a trend for farms in which the silos were not disinfected compared with farms where disinfection was carried out (62.5% compared with 35.3%, $P < 0.1$, Table 3). These results suggest that *B. cereus* group spores could persist in the silo and the feeders, develop in the feed and disperse in the house environment, resulting in eggshell contamination.

According to Mayes and Takeballi (1983), Board and Tranter (1995) and Protais *et al.* (2003), eggshell contamination could come from dust, soil or faeces, and the presence of bacteria could be ascribed to their tolerance to dry conditions. Gast *et al.* (1998) and De Reu *et al.* (2005) also identified airborne transmission as the main route of contamination in the house environment. In dairy cow houses, the air is also considered as a source of contamination, even if the number of aerial spores is too low for airborne contamination to be of major importance (Christiansson *et al.*, 1989; Te Giffel *et al.*, 1997). Vissers *et al.* (2007) showed that the dominant route of milk contamination by *B. cereus* was from feed, via faeces, to farm tank milk. Magnusson *et al.* (2007a) also showed a positive correlation between the *B. cereus* spore content in feed and in faeces.

These effects of the environment on eggshell contamination were substantiated by the effect of the hygienic practice of the farmer on the level of contamination by *B. cereus* group cells and, particularly, when the farms were composed of several buildings: the fact that the farmer circulated between several buildings and that he used an inefficient sanitary wall represented a significant risk for eggshell contamination ($P < 0.05$ and $P = 0.1$, respectively, Table 3). Davies and Breslin (2003) noted an increasing risk of contamination by *S. enteritidis* in farms with several layer houses. According to the experimental results, the farmer was another contamination vector. The putative cross-contamination between the buildings was apparently reduced by the use of an efficient sanitary wall.

The type of disinfection tended to be associated with the risk of contamination ($P < 0.1$, Table 3): 56.5% of the farms using a spray treatment were positive as opposed to the 37.5% for farms using a steam treatment. The steam treatment may be more efficient in killing *B. cereus* group spores than the spray treatment. This observation is important because most contaminations originate from improperly cleaned and disinfected poultry houses.

There was a significant effect of the origin of the pullets ($P < 0.05$, Table 3): 58.3% of the farms using pullets originating from the same flock were positive as opposed to the 28.6% positive results for farms using pullets originating from different flocks. Unfortunately, we have no hypothesis to explain this result showing it is possible that these flocks may already have been contaminated before entering the farm.

Several recent studies on the influence of the change of the housing system on eggshell contamination following the EU 1999/74/EC Directive banning conventional cages in 2012 have been published (Protais *et al.*, 2003; De Reu *et al.*, 2005, 2006; Wales *et al.*, 2007). De Reu *et al.* (2006) and Protais *et al.* (2003) observed higher counts of aerobic bacteria on eggshells for alternative than for conventional systems. By contrast, no difference was observed by De Reu *et al.* (2005) concerning the contamination by Gram negative bacteria. Although some of the significant effects described in our study were linked to production practices, the housing characteristics, that is conventional versus furnished cages, number of hens per cage and number of cages and floors per house, had no influence on the level of eggshell contamination by *B. cereus* group bacteria. Concerning the age of the hens, our results corroborated those of De Reu *et al.* (2005) and Protais *et al.* (2003), who showed that this parameter had no influence on eggshell contamination by mesophilic bacteria.

This study is the first investigation of the risk factors for eggshell contamination by *B. cereus* group bacteria at the production level. Consistent with numerous studies dealing with this type of contamination in the environment of milk production, suggesting that soil, faeces, bedding, feed, air and the milking equipment are the putative sources of contamination ((Magnusson *et al.*, 2007a, 2007b; Vissers *et al.*, 2007; Bartoszewicz *et al.*, 2008), the results showed that dispersion by dust, dry manure removal, feeders and the farmer himself were likely the predominant routes of eggshell contamination by *B. cereus* group cells. In the area of egg production, De Reu *et al.* (2005) showed that eggshell contamination by *Salmonella* also depends on the flooring and feeding systems. Farmers should then take measures that minimise the transmission of spores by ensuring low initial

contamination levels in the feed and by preventing dispersion and persistence of *B. cereus* group cells in the farm environment. The research highlights the benefit of several management procedures, including the limitation of dust formation from the manure and feeding practices and an efficient disinfection of the silo, the sanitary wall and the houses.

From the public health and economical points of view, the monitoring and control of this type of microorganism are important for the egg breaking industry. However, the ubiquitous nature of spore-forming bacteria in general, their property of heat resistance and their capacity to settle and multiply on various materials render this type of microorganism difficult to eradicate. Identifying the origin of contamination at the farm level, and during egg transport and egg product processing, is essential to better control these bacteria, throughout the farm-to-fork continuum, and to initiate the best intervention strategies.

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