Free-range and organic farming: Eggshell contamination by mesophilic bacteria and unusual pathogens

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Primary Audience: Quality Assurance Personnel, Researchers

SUMMARY

The high incidence of foodborne illnesses caused by the consumption of eggs in industrialized countries is the main reason we decided to determine the microbial load on the surface of eggshells from free-range and organic farming. The objective was to compare which was better for ensuring the least possible health risk to the consumers, focusing on consumption of raw eggs by immune-compromised people. Bacteria come from the intestine of the animal or subsequent contamination. When eggs are cracked, bacteria from the shell reach the yolk and the albumen, and grow during manipulation and preservation, causing foodborne diseases in consumers. Microorganisms such as *Salmonella*, *Listeria monocytogenes*, *Campylobacter*, *Enterobacteriaceae* (including *E. coli* serotype O157: H7), *Staphylococcus*, *Enterococcus*, and mesophilic aerobic bacteria, were examined. The presence of bacteria on eggshells depends on hygienic conditions of the farming and packaging industries. Hygienic measures, such as strict cleaning and disinfection of surfaces in contact with eggs in packaging industries, would be a protective factor to minimize the contamination of eggshells. The total absence of pathogens demonstrates the relevance for human consumption of eggs coming from both free-range and organic farms, though YOPI (young, old, pregnant, or immune-compromised) people preferably should cook eggs in which bacteria contaminating the outer surface are killed.

Key words: eggshell, free-range, organic, microbiology, pathogens

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DESCRIPTION OF PROBLEM

In Europe, there are approximately 363 million laying hens producing 6.9 million tons of eggs each yr [1] by 4 production systems: organic, free range, barn, and caged [2, 3]. In both organic and free-range farming, hens have access to an outdoor area during the d, whereas in the other types of breeding, hens are kept in sheds, provided with feed, and do not have much space to move around. Moreover, in organic farming, the use of synthetic chemicals is forbidden, including synthetic hormones and antibiotics, and organic feeds are 100% organic [4]. Benefits of organic and free-range farming include: greater comfort for the hens, easier inspection procedures, and greater environmental quality, resulting in quality products with lower possibility of contamination from pathogens [4, 5]. Eggshells are normally contaminated by bacteria during passage through the cloaca and from contact with contaminated surfaces. When eggs are cracked,

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due to penetration through shell microfractures [5], which can occur because of egg aging and during storage, transport, and sale, bacteria can be transferred to the yolk and albumen, becoming a potential hazard for consumers. Sanitization of eggs and egg products occurs when the former are hard-boiled, and the latter are cooked at least at 71°C for one minute [6]. Therefore, the consumption of cooked eggs or egg products does not constitute a health risk for immune-competent individuals or for individuals categorized as YOPI (young, old, pregnant, or immune-compromised). Eating raw eggs could, however, cause foodborne disease, especially in the class of old people. In 2013, a total of 5,196 foodborne outbreaks, including waterborne outbreaks, were reported in the EU; overall, 43,183 human cases, 5,946 hospitalizations, and 11 deaths were reported [7]. As in previous yr, the most important food vehicles in outbreaks were eggs and egg products (18.5%) followed by mixed food, fish, and fish products [7]. Most foodborne outbreaks were caused by *Salmonella* (22.5% of all outbreaks), viruses (18.1%), bacterial toxins (16.1%) (produced by *Bacillus*, *Clostridium*, and *Staphylococcus aureus*), and *Campylobacter* (8.0%). However, while the number of *Salmonella* and *Campylobacter* outbreaks decreased during the last 6 yr, the number of outbreaks due to bacterial toxins increased during the same time [7]. Microorganisms associated with live poultry are located both on the surface of the birds (skin, feet, feathers) and in their gastrointestinal tract. The numbers and species depend largely on the environmental conditions under which the hens grow (soil, feed, litter, drinking water, insects, rodents, wild birds, and farm workers), and it includes a large variety of microorganisms, such as *Pseudomonas, Acinetobacter, Micrococcus, Enterobacteriaceae, Enterococcaceae, Staphylococcus, Bacillus, Clostridium,* molds, yeasts, and pathogens such as *Salmonella, Yersinia*, and *Campylobacter*. Contamination of animals increases because of further distribution of microorganisms from bird to bird, primarily through contact with fecal material [8]. Hence, microorganisms on egg surfaces occur because of their presence in the intestine of the animal, or because of secondary contamination by the animal's own and other animals' feces, or due to manipulation events during packaging in industries.

Due to the frequent use of eggs, both in domestic and restaurant cooking, and the high incidence of foodborne illnesses caused by the consumption of eggs and egg products prepared with raw eggs, we decided to determine the microbial load of the surface of eggshells from free-range and organic farming sold in Italian supermarkets.

According to EC Regulation 589/2008 [3], adopted in Italy in December 2009 [9], the manufacturer is obliged to print a code on each eggshell for the purpose of traceability: The first number indicates the type of farming (0 organic; 1 free range; 2 on ground; 3 cage); the next 2 letters indicate the country of origin; the 3 numbers following indicate the ISTAT (Italian National Institute of Statistics) code of the town where the farm is located, then the next 2 letters indicate the province of the farm; the final 3 numbers indicate the name and place of the farm. Thanks to the code printed on the eggshells, we also tried to identify the stage within the production chain at which the contamination most probably occurred, to determine whether the responsibility for the contamination was attributable to the manufacturing company (farm of origin) or to the packaging industry. Finally, we tried to determine, among 5 brands, and between the 2 farming systems examined, which combination of brand and system of farming was less contaminated — this with the aim of ensuring the least possible health risk to consumers who eat raw eggs or raw preparations, especially regarding YOPI people who have reduced immunocompetence and can develop a foodborne disease caused by a very low minimal infective dose (**MID**).

MATERIALS AND METHODS

Sample Collection

Samples were obtained from 2 supermarkets selling selected brands of eggs throughout Italy. A total of 300 eggs was analyzed: 120 were from free-range farms [60 from supermarket 1 (brand 1) and 60 from supermarket 2 (brand 2)]; 180 were from organic farms [60 from supermarket 1 (brand 3) and 60 from supermarket 2 (brands

Table 1. Classification of examined eggs.

Farm	Farming[1] system	Brand	Supermarket
A	FR	1	1
B	FR	1	1
C	FR	1	1
D	FR	2	2
E	O	3	1
F	O	3	1
F	O	4	2
G	O	5	2

[1]FR = free range; O = organic.

4 and 5)]. Samples were collected in germ-free conditions from their packaging, then placed in sterile polyethylene carrier bags, refrigerated at 4°C, and analyzed on the d of collection, at least 15 d before the expiration date. For our study, we collected only category M (medium) and L (large) eggs [3]. The code printed on the eggshells was used to trace 7 farms (Table 1).

Microbiological Analysis

Samples (one whole egg) were blended for 60 s in 10 mL of 0.1% buffered peptone water. Decimal dilutions were carried out using the same diluent and were used to inoculate agar media. Culture media were all from Thermo Scientific [10]. Results were expressed in cfu/cm^2 of eggshell surface and detected or not detected on the whole egg surface, depending on bacteria.

Mesophilic aerobic count (**MAC**) was obtained on duplicate plates of tryptone glucose yeast agar incubated at 30°C for 72 h, according to the method described by ISO 4833–1 [11]. *Enterobacteriaceae* count was obtained on crystal violet neutral red bile lactose agar incubated at 37°C for 24 h in conformity with ISO 4832 [12]. *Escherichia coli* count was obtained on TBX medium incubated at 44°C for 48 h in conformity with ISO 16,649–2 [13]. *Escherichia coli* O157:H7 count was obtained on sorbitol McConkey's agar incubated at 37°C for 48 h in conformity with ISO 16,654 [14]. Identification of *E. coli* serogroup O157 was made through *E. coli* O157 Latex Test [10]**.**

Detection and identification of *Enterococcus* spp. were performed as described by Pesavento et al. [15] on Slanetz and Bartley agar incubated for 24 h at 37°C. After incubation, suspected colonies of *Enterococcus* spp. were transferred to tryptone soya agar and incubated for 24 h at 37°C, and identified through Gram stain, catalase production, and rapID STR [10].

$$2\pi\alpha^2 + \pi\alpha\left(\frac{\beta^2}{\sqrt{\beta^2-\alpha^2}}\cos^{-1}\frac{\alpha}{\beta} + \frac{\gamma^2}{\sqrt{\gamma^2-\alpha^2}}\cos^{-1}\frac{\alpha}{\gamma}\right)$$

Figure 1. Surface area formula for an egg (α = equatorial radius, β = short polar radius, γ = long polar radius).

Staphylococcus aureus count was obtained on Baird Parker agar incubated at 35°C for 48 h in conformity with ISO 6888–1 [16]. Identification of suspected colonies was made by means of Api Staph. [17].

Salmonella detection was performed on Hektoen agar incubated at 37°C for 24 h, in conformity with ISO/DIS 6579–1 [18] after 2 selective enrichment phases, the first in buffered peptone water incubated at 37°C for 18 h, the second in Rappaport-Vassiliadis incubated at 41.5°C for 24 hours.

Campylobacter detection was performed on charcoal cefoperazone deoxycholate (**CCD**) modified agar incubated at 41.5°C for 48 h, in conformity with ISO 10,272–1 [19] and after selective enrichment in Bolton broth incubated at 41.5°C for 48 hours.

Listeria spp. strains were isolated in accordance to ISO/DIS 11,290–1 [20], after 2 selective enrichment phases in half Fraser and Fraser broth incubated, respectively, at 30 and 37°C for 18 hours. The broth (10 μL) was streaked onto Palcam agar with Palcam selective supplement and incubated for 24 h at 37°C. The suspected colonies were characterized by Gram stain. Gram-positive colonies were tested for hemolysis on Columbia blood agar, catalase [21], and species identifications were made with the API Listeria kit [17].

Calculation of the Eggshell Surface

The calculation of the egg surface area was made through the application of the formula of Figure 1 [22].

Statistical Analysis

Microbial counts were expressed in cfu/cm^2 of eggshell surface. The standard descriptive

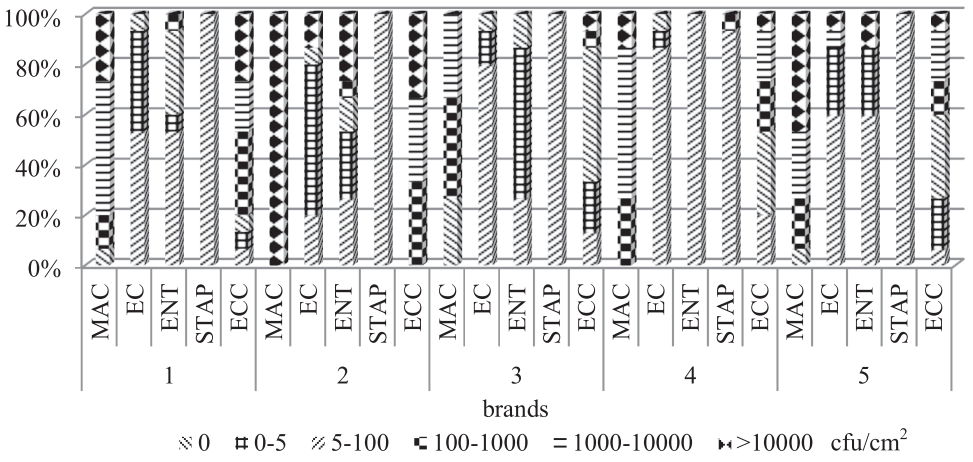

Figure 2. Bacterial load of eggs from the 5 brands (60 examined samples for each brand). Classes of contamination are expressed in cfu/cm^2 of eggshell. MAC, mesophilic aerobic count; EC, *E. coli;* ENT, *Enterobacteriaceae;* STAP, *S. aureus;* ECC, *Enterococcus* spp.

statistics of the contamination (means) and comparison tests were made using Epi Info 3.5.1. 2008. The frequencies were compared using the chi-squared or Pearson tests with a significant level of P-value < 0.05.

RESULTS AND DISCUSSION

Surface of the Eggs

The surface of the eggs sized M was estimated at 66.3 ± 3.86 cm^2; the surface of the eggs sized L was estimated at 74.22 ± 2.54 cm^2.

Microbial Load of the Eggshells

Most contamination was due to MAC (Figure 2). *Escherichia coli* and *Enterobacteriaceae* showed the same trend. Most samples were not contaminated, or bacterial contamination was <5 cfu/cm^2. In the few samples contaminated, the concentrations were high. *Escherichia coli* was present in high concentration in 8 samples of brand 2 (about 10^5 cfu/cm^2) and 8 of brand 5 (higher than 10^3 cfu/cm^2). *Enterobacteriaceae* contaminated mostly brands 2 and 5, in 16 and 8 samples, respectively, with high bacterial content (10^4 cfu/cm^2).

Staphylococcus aureus contamination of the shell might occur during passage through the cloaca or after it has been laid [23]. There are many episodes of food poisoning caused by eating eggs contaminated by *S. aureus* [24, 25], but of our 300 egg samples, only 4, of brand 4, resulted in contamination at a mean concentration of $2.75 \pm 1.7 \times 10^2$ cfu/cm^2. This microorganism could cause food poisoning thanks to the production of enterotoxins during its growth at temperatures between 10 and 40°C in raw food preparations or when yolk is manually separated from albumen, making contact with the contaminated shell.

Enterococci were present in high concentrations in all samples of the 5 brands analyzed, and particularly in brands 1 and 2. These bacteria were more present in free-range eggs than in organic eggs. While the other species were rarely isolated, the eggs were more frequently contaminated by *E. faecalis* (30.6%), *E. faecium* (46.9%), or by both species (17.34%) (Table 2), all in high concentrations ($>10^3$ cfu/cm^2). The

Table 2. Number of eggs contaminated by *Enterococcus* species.

	Free range		Organic		
	Brands				
	1[1]	2	3[1]	4[1]	5[1]
Enterococcus casseliflavus	n.d.	2	2	n.d.	n.d.
Enterococcus durans	4	0	4	2	4
Enterococcus gallinarum	10	2	n.d.	10	12
Enterococcus faecalis	8	20	2	10	20
Enterococcus faecium	26	18	18	18	12

[1]n.d. = not determined.

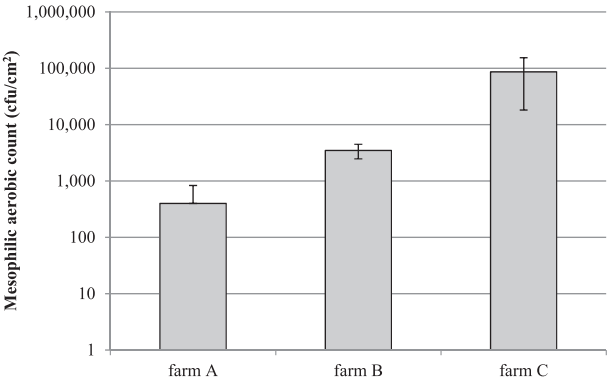

Figure 3. Mesophilic aerobic count of brand 1 samples coming from farms A, B, and C. Data represent the mean of 16, 24, and 20 samples and their standard errors, respectively.

fact that these bacteria were more present in free-range eggs than in organic eggs is in accordance to the breeding characteristics. In the first type, hen density is higher than in the second one (9 hens/m^2 and 6 hens/m^2, respectively), although both systems provide opportunities for the hens to move around on grass, eating insects and worms, and benefiting from fresh air and natural light. *Enterococci* are responsible for opportunistic infections, which may be dangerous, especially for the most sensitive subjects [26]. Furthermore, the frequency of antibiotic-resistant isolates of *Enterococcus* species, especially in nosocomial strains, is increasing worldwide [14,27–29]. Antibiotic-resistant *Enterococci* may cause possible failure of therapeutic treatment in case of infections, such as severe urinary tract diseases, bacteremias, and endocarditis [30].

Comparison Among Brands and Farming Systems

Due to the fact that mesophilic aerobic bacteria were present in all samples, we decided to use these values to compare contamination of eggs of different brands and farming systems. Comparing the microbiological quality of eggs from brand 1 and 2 of free range (Figure 2), we observed that the eggs of brand 2 had a hygienic quality significantly inferior to the eggs of brand 1, showing the poor hygiene standards of the staff engaged in farm D and/or during the egg-packing process for supermarket 2. The eggs of brand 1 were from farms A, B, and C. From the results (Figure 3), we observed that A produced less contaminated eggs than B, which produced less contaminated eggs than C. These differences are to be attributed to the presumably high level of hygienic practices and cleanliness of packing rolls of the packaging industry (brand 1).

All the eggs of brand 2 and a high percentage of eggs of brand 5 were highly contaminated ($P = 0.80$) (Figure 2). The two brands are sold only by supermarket 2; therefore, despite coming from different farms, they presumably share the same packaging industry, which is poor in hygienic sanitization (Table 1).

Averages of bacterial loads of the 60 eggs of brand 4 coming from farm F and of the 32 eggs of brand 3, also coming from farm F (Figure 4), were significantly higher ($P < 0.05$) for the former than the latter. This statistical difference may be caused by failure to comply with hygiene standards during egg packaging (e.g., roller cleaning) by the industry (brand 4) that provides eggs to supermarket 2. Mesophilic aerobic count of samples from farms E and F, both of brand 3, were statistically different too, so we may deduce that in this case the different microbiological quality of eggs is not due to contamination during packaging by the industry (brand 3), but only to different hygienic conditions of the farms.

Van Hoorebeke et al. [31] found significantly higher *Salmonella* infections in flocks in housing systems in winter compared to the other seasons of the year, due to the fact that in winter hens are kept inside because of wet and cold weather

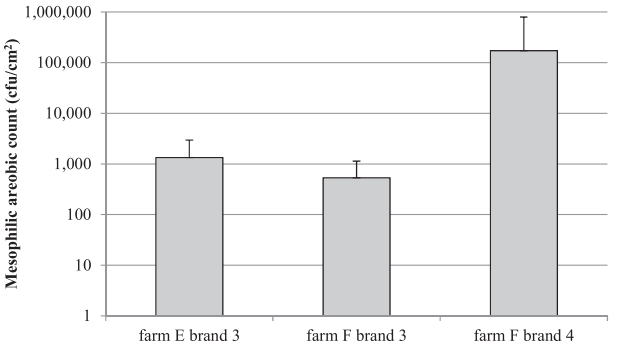

Figure 4. Mesophilic aerobic count in cfu/cm^2 of brands 3 and 4 samples coming from farms E and F. Data represent the mean, respectively, of 28, 32, and 60 samples and their standard errors.

conditions, causing low air quality [32, 33] and high density of animals, which are well-known risk factors for the spread of *Salmonella* on farms [34, 35]. Despite the fact that hens of organic and free-range systems are outdoors and sanitization of the "floor" is impossible, eggs from these types of farms are of high quality.

Escherichia coli O157: H7, *Listeria monocytogenes, Campylobacter*, and most *Salmonella* serovars are adapted pathogens that do not cause illnesses in animals but can be pathogenic for humans when transferred through feces to eggs [36, 37]. We, therefore, thought necessary the search for these pathogens. Messelhäusser et al. [37] found very low prevalence of the *Campylobacter* and *Salmonella* (4.1 and 1.1%, respectively). In our sampling, we did not find any *Campylobacter* or *Salmonella* contaminated eggs. Despite the fact that *Salmonella* was rarely found in eggs in Europe (0.03%) [7], eggs and egg products are the most important sources of foodborne *Salmonella* outbreaks because eggs are used in the preparation of many foods (e.g., bakery products including pastries, meat pies, sauces and dressings, sweets, and pasta) and in several homemade dishes (e.g., mayonnaise, tiramisu, and ice cream). Moreover, in such products, eggs are often used raw or only slightly heated. The frequency of *Salmonella* occurrence (16%) observed on eggshells by Guzmàn-Gòmez et al. [38] was much higher than what we reported (0%).

Poultry is considered a major reservoir for human campylobacteriosis [39]. In Europe, in 2013, at retail, *Campylobacter* was detected in 26.4% of the broilers tested, causing at least 64.8/100,000 confirmed cases [7]. These bacteria are symbionts in the intestine of the hens and can contaminate broilers during slaughtering and may be expelled through the cloaca in concentrations of up to 10^{10} cfu per gram of feces [40], thereby contaminating eggshells. We did not find any data on contamination of the egg surfaces. Fonseca et al. [41] found that, out of the 17.8% of hens positive for *Campylobacter* from cloacal swabs, no egg was positive, as we found. This is probably due to the fact that *Campylobacter* is a microaerophilic bacterium, and thus it cannot grow under normal atmospheric oxygen conditions.

Research of *L. monocytogenes* is also unusual on eggshells. This microorganism is widespread in the environment and, therefore, a wide range of different foodstuffs and animals can be contaminated, even hens (1.6%) [7]. In fact, this pathogen has been isolated from feces, body fluid, and oviducts of asymptomatic laying hens and can contaminate eggs during the passage through the cloaca, collection, and processing. There have been few documented foodborne illnesses caused by *L. monocytogenes* following the consumption of eggs or egg products [42], and the data on the presence of this species on eggshells [43, 44] are in accordance with ours (0%).

In recent years, *E. coli* O175: H7 prevalence in food is increasing (mostly in vegetables and meat) and in hospitalized cases had a fatality rate of 0.36% (13 deaths) in 2013 [7]. As all the other bacteria coming from hen cloaca [45], *E. coli* O175: H7 can be present on egg surfaces and potentially

contaminate albumen and yolk when cracked, causing serious diseases, due to its very low MID (<10 viable cells) [46]. We did not find any data in literature to compare with our results for this bacterial species (0%).

Due to the fact that we did not isolate any pathogen, we can assume that eating cooked and raw eggs does not constitute a serious risk to the health of immune-competent consumers. For subjects in the YOPI category, and in particular for the immune-compromised, there may be a risk in the consumption of raw food preparations [47] in both of the 2 types of farming taken into consideration (free range and organic), mainly due to the high presence of *Enterococci*.

CONCLUSIONS AND APPLICATIONS

1. Interpreting the data collected, it was not possible to deduce which type of farming, organic or free range, produced fewer contaminated eggs; we could only observe that brands 1 and 3, purchased at supermarket 1, had fewer contaminated eggs than brands 2, 4, and 5, purchased at supermarket 2.
2. Thanks to the comparison between the microbial load of eggs coming from the same farms, we can attribute the different levels of microbial contamination to the packaging companies (brands), where eggs are treated with different hygiene procedures.
3. In the packaging industry, strict cleaning and disinfection of the surfaces in contact with eggs are necessary to minimize the contamination of eggshells.
4. In the future, a study of the contamination of eggshells from other farming systems will be conducted.
5. High density of animals could be a real risk factor for the spread of pathogens on farms.
6. Due to the fact that organic and free-range systems are outdoors and sanitization of the "floor" is impossible, eggs from these types of farms are of high quality.
7. The total absence of pathogens, such as *Salmonella* spp., *Escherichia coli* 0157: H7, *Campylobacter* spp., and *Listeria monocytogenes,* demonstrates the relevance for human consumption of eggs coming from both free-range and organic farms. Eggs are media favorable to bacterial proliferation (coming from outside) because of their richness of nutrients, so YOPI people should consume preferably cooked eggs, even more so if we consider the high concentration of *Enterococci* we found in this study.

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